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Genetic and ecological analysis of the putative natural hybrid zone formed Banksia robur Cav. and Banksia oblongifolia Cav.

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Genetic and ecological analysis of the putative natural hybrid zone formed by *Banksia robur* Cav. and *Banksia oblongifolia* Cav.

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A thesis submitted in fulfilment of the requirements for the award of the degree of

Doctor of Philosophy

from

The University of Wollongong

by

Suzanne Margaret Schibeci B.Sc (Hons) UNSW

Department of Biological Sciences

1994

Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the requirements of the degree of Doctor of Philosophy. The work described in this thesis was carried out by me and has not been submitted to any other university or institution.

Suzanne Schibeci
April 1994

Abstract

Natural hybridization provides the opportunity to study the processes involved in speciation, since hybridizing populations are thought to represent some intermediate stage between a common ancestral population and the complete divergence of two species. The presence of hybrids within a population indicates that reproductive isolation has at some stage been incomplete. Therefore, the determination of the current level of gene flow can indicate the degree to which speciation is complete. Further, the fitness of the hybrids in comparison to that of the parent species can indicate the mode of formation and maintenance of the hybrid zone: either (i) a genetic/morphological cline can form in association with an environmental cline if the species are suited to conditions on different extremes of the cline or if the hybrid are as fit as the parents in the ecotone (environmental cline) and (ii) a cline may be formed as a result of an equilibrium formed between the constant gene flow into, and the selection against hybrids within, the hybrid zone (tension zone).

Banksia robur Cav. and *Banksia oblongifolia* Cav. form the most commonly reported hybrid zone within the genus. The two species are morphologically different and are also closely associated with very different soil water regimes. Morphologically intermediate individuals, present in regions where the two species coexist, are thought to be hybrids.

This hybrid zone, therefore, provides the opportunity to compare the "environmental cline" versus "tension zone" modes of hybrid zone formation, because while the *Banksia* hybrid zone seems to be associated with an environmental cline, there is also evidence that hybrids are selectively disadvantaged. In order to make this comparison, there are three general aims of this thesis: 1. To describe the "parental species", hybrids, and the hybrid zones as they are at present; 2. To determine if hybrids are selected against at some stage

of development; 3. To determine the present potential for hybrid production, through the determination of the extent of reproductive isolation within the populations.

A genetic survey within allopatric populations of *B. robur* and *B. oblongifolia* showed that there were some useful genetic differences between the two species. Of an electrophoretic survey of 32 enzymes, none was variable in stands of *B. robur*. Four loci (*Adh*, NSdh, *Sod* and *Gdh*) were variable for *B. oblongifolia*. Three of these loci (*Adh*, *Sod* and *Gdh*) were useful in differentiating the two species: the alleles for which *B. robur* were fixed at these loci, were the least common *B. oblongifolia* alleles. A genetic hybrid index was constructed using the variation in the allopatric populations of the species. Each plant from the pure *B. robur* had genetic hybrid index scores (GHIS) of 0. "*B. robur* alleles" were also present in small frequencies even in the allopatric populations of *B. oblongifolia*, the pure *B. oblongifolia* plants therefore scored GHISs of either 5 or 6. Nine leaf characters were quantified for each plant in the allopatric population, and an additional five inflorescence characters were added when inflorescences were available. Again a hybrid index was constructed from these morphological characteristics. A comparison of a plant's morphological hybrid index score (MHIS) and its GHIS revealed a good association of GHIS with morphology.

The hybrid indices developed using the allopatric populations of the species were then applied to the plants within the two hybrid zones. The plants within the hybrid zones encompassed the range of scores possible on both the genetic and morphological hybrid indices, indicating that not only are parental species present in the hybrid zones, but these are hybridizing and forming plants of intermediate morphology and genotype. Further, plants with genotypes between parental and hybrid genotypes indicated that there is backcrossing and introgression occurring extensively within the hybrid zone. Using multivariate analysis, the morphology could separate plants with the parental scores of 0 from 5 and 6. The plants of hybrid origin were intermediate to the parental plants, but

separation of the hybrid genotypes (i.e. GHISs of 1, 2, 3 and 4) by morphology was not possible, even when multivariate analysis was employed.

The spatial arrangement of the individuals within the hybrid zones was genetically complex. In order to simplify this spatial arrangement, the frequency of the *B. robur* alleles were determined along a transect through each population. This approach provided the opportunity to observe the genetic cline through the hybrid zones.

The cline obtained using the mean frequency of the three loci along the transect was used to determine several parameters describing the cline: cline width (w), the rate of decay of the tails (θ) and the strength of the barrier to gene flow set up by the cline (B). The clines in both sites were narrow (~25 - 60 metres), and are amongst the narrowest reported. The value of θ for the *B. oblongifolia* side of the cline was extremely small (0.01-0.04), indicating that there is substantial introgression of the *B. robur* alleles into the *B. oblongifolia* genome, but not vice versa. Similarly, this asymmetrical gene flow was supported by a high value of B_0, indicating a relatively weak barrier to *B. robur* alleles going into the *B. oblongifolia* genome. There was, however, a strong barrier to *B. oblongifolia* alleles going into the *B. robur* genome.

The coefficient of linkage disequilibrium, determined within the pure stand populations of *B. oblongifolia*, and for the plants within the hybrid zones showed that, while there were no significant linkage association between loci within the pure stand *B. oblongifolia*, significant linkage was detected between loci within the hybrid zone populations. Similar results have been obtained in many other hybrid zones, where significant linkage between loci increases towards the middle of the cline, because of the constant influx of parental genotypes.

Cline width, the extent of introgression and the linkage between loci within the hybrid zone were used to determine the dispersal distance of the alleles within the hybrid zone

and the selection coefficients within and outside the cline. Dispersal distance was fairly long (100 - 450 metres) compared to the cline width, indicating long distance gene flow. Selection against the *B. robur* alleles within the hybrid zone was extremely strong (~100%), whilst outside the cline, the same alleles were subject to fairly weak selection (~3%). Strong selection against hybrids within the centre of the cline was supported by a significant deficit of heterozygotes within the hybrid zone (while the pure populations of *B. oblongifolia* were in Hardy-Weinberg equilibrium).

There was, however, no significant difference between the parental and hybrid plants in plant vigour or fertility, nor was there any spatial transition in increased vigour or fertility in either the parental or hybrid individuals. This indicates that perhaps the characters chosen to represent fitness of the plants were responding to very complex environmental conditions.

The current potential for interspecific gene flow was assessed through observations of flowering time and foraging behaviour of probable pollinators. Despite having distinct peak flowering times, there was a period of overlap in the flowering time between the two species, providing some opportunity for interspecific pollination. Further, there was overlap in flowering time between the parental morphs and the hybrids, providing some opportunity for backcrossing and introgression. However, the majority of infructescences were formed from inflorescences that flowered close to the peak flowering time of the morph. Further, the potential proportion of seed produced as a result of inter-specific pollination indicated that hybrid production may be, in fact extremely limited. Only around 1% of fruits produced by *B. robur* infructescences could potentially have been the result of pollination by *B. oblongifolia*, while around 10% of *B. oblongifolia* fruits were potentially the result of pollination by *B. robur*.

Hybrid production did not seem to be limited by pollinator behaviour. An examination of the potential pollinators within the *B. robur*/*B. oblongifolia* system showed that the plants

were visited most often by birds: particularly the New Holland honeyeater and the red wattlebird. These species of bird are opportunistic foragers and, in this study, were observed to visit both the species and the hybrid in succession, providing the opportunity for interspecific pollen exchange, and exchange between the species and the hybrids.

A comparison was made of the distance flown by the birds between successive inflorescences, and the distance over which a rare allele, the b allele on NSdh was dispersed was made. This showed that the distance flown by the birds both between inflorescences and in a whole foraging bout, were in the order of 10 times greater than the gene flow distance inferred from the distance moved by the NSdh b allele. This may suggest that bird movements are not necessarily radial from the pollen source, which also brings pollen-carryover into play as a crucial factor in pollen dispersal in this system. The values obtained for the dispersal variance using birds and the NSdh b allele were further contrasted with the values obtained for σ^2 using the cline shape parameters. The values obtained for σ^2 using the cline shape were much larger than those obtained using the NSdh, but were comparable to those using bird flight distances. This may indicate two things: that, again, pollen-carryover has a large role within this system, and that the NSdh is not selectively neutral, being affected by the high level of selection detected within the cline calculated using the cline shape parameters.

The neighbourhood area (N_A) and the effective neighbourhood size (N_e) were calculated using the NSdh b allele. N_e was estimated to be 1-2 individuals, while N_e estimated from bird flight distances was 6 for between inflorescences and 11 for distances flown within a foraging bout. This suggests that the gene flow within this system is very limited, and therefore that interspecific gene flow is potentially limited and slow, a suggestion which is supported by the determination of the pollen pool heterogeneity for the species, where significant departures from random mating were found in the hybrid zone populations.

There is every indication that the potential for hybridization between the species is high, but the realized hybrid production was lower than would be expected. The hand pollination experiment resulted in the production of no hybrid seed, while the natural hybrid seed production was lower than what would be expected from both the proportion of hybrid plants within the hybrid zones, and the potential amount of pollen exchange determined from flowering synchrony and random mating. The results of the field planting experiment suggest that there may be some post-dispersal selection.

This study indicates that the origin of the hybrid zone formed by *B. robur* and *B. oblongifolia* is complex. There is evidence supporting that it is maintained through the balance between dispersal and selection against hybrids (a "tension zone"), while there is also obvious association with environmental gradients. The maintenance of the hybrid zone is probably achieved through a combination of both.

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Chapter One

General Introduction

1.1. Hybridization and hybrid zones

Hybridization between organisms has long held interest for humans. Reports of fantastic creatures in antipodean regions of ancient civilisations were the basis of many mythological stories and adventurers' tales, and included claims of bizarre offspring resulting from the union of organisms from different classes, phyla, and even kingdoms (reported, for example, by Pliny the Elder [Rackham 1940]). Although these creatures were largely products of fertile imaginations, the fascination with hybridization of more closely related organisms still exists. Controlled crossing between species of plants and animals has been used for centuries in the development of different varieties for use in horticulture, agriculture and as pets. However, although Darwin (1859) considered the role of natural hybridization in speciation, it has only been since the development of electrophoretic techniques that there has been an objective method that is unequivocally liked to genetics for detection of hybrids. Together with observed morphological and behavioural differences, characterization of genetic differences enhances the positive identification of instances of natural hybridization.

1.1.1. Definition of hybrid zone

Endler (1977) defined a hybrid zone as a narrow belt of increased variability in fitness and morphology, separating distinct groups of relatively uniform populations. In common usage, the term "hybrid zone" has been equated to "cline", though Barton and Hewitt (1981 and 1985) preferred the term "hybrid zone" to refer strictly to clines that are maintained by a balance between dispersal and selection. Both the Endler and the Barton and Hewitt models adhere to this definition, but the role played by selection in their models differ. Endler (1977) suggested that clines are formed as a result of genotype-

specific responses to environmental factors that are geographically variable. Barton and Hewitt assumed that many hybrid zones are independent of environmental factors, and that there is a dynamic equilibrium between dispersal and hybrid fitness. There are examples recorded within the literature fitting both models (see Hewitt 1989),

Natural hybrid zones provide the opportunity to study processes involved in speciation, because hybridizing species are thought to represent a stage just short of complete specific divergence (Hewitt 1988). The presence of hybrids within a population only indicates that reproductive isolation has at some stage been incomplete. Measuring the current level of hybridization can indicate if complete divergence has been achieved. Further, the fitness of the hybrids established within the hybrid zone can indicate if complete speciation is inevitable. The study of hybridization between two species should, therefore, encompass three broad areas: 1. the confirmation that putative hybrids are formed as a result of cross fertilization of the species; 2. determination of the current level of gene flow between the two species; and 3. an assessment of the fitness of the hybrid individuals within the hybrid zone.

1.1.2. Occurrence of hybridization

Hybridization is described by Stebbins (1959) as the "crossing between individuals belonging to separate populations which have different adaptive norms". Recognition of the products of this "crossing" in nature has often been based on the intermediacy of morphological and/or behavioural traits (Baker 1947). However, the occurrence of individuals exhibiting intermediacy in these traits in association with two groups of distinct organisms, does not necessarily indicate interspecific hybridization. Intermediacy of certain individual organisms, and indeed the formation of a morphological cline, can be linked closely to environmental variation, as a result of phenotypic plasticity (Bradshaw 1965). Determination of isozymes or DNA would contribute towards eliminating the possibility of phenotypic plasticity, although it is possible (however remote) that intermediacy in isozymes can arise through independent evolution.

The best way to confirm the occurrence of hybridization in nature is to compare morphological, behavioural and genetic characters of the putative natural hybrid with artificially produced hybrids (Baker 1947). Natural hybridization is further supported by the direct observation of interspecific gametic transfer (e.g. in plants, interspecific foraging of pollen vectors [Levin 1979]). However, it is often not possible to use all these criteria in all systems, therefore inferences need to be made from what can be measured and circumstantial evidence.

1.1.3. Gene flow and reproductive isolation

A crucial factor in the formation and maintenance of hybrid zones is the extent of gene flow between species or types. Endler (1977) defined gene flow as the movement of genes among and their establishment in unlike gene pools, and stresses the difference between "gene flow" and "gene dispersal" or "gene migration", which have often been used synonymously (Levin & Kerster 1974). Gene dispersal involves fairly small distance, non-directional movements by individual organisms (or in the case of pollen, male gametes), on a continuous temporal scale and as a result of normal activities, while gene migration can be considered as sporadic, relatively long distance movements in the same direction (Endler 1977). Neither gene dispersal nor migration necessarily results in the incorporation of the "moving" gene into another gene pool. For gene flow to be effective, the gene must be dispersed, and established within another population (at least in the short term), leading to successful breeding.

Normally, interspecific gene flow is restricted by specific mate recognition systems (Paterson 1982), which can come into effect at one of a number of stages of the organism's development: (i) premating, and (ii) postmating, including establishment. Isolation at the pre-mating stage includes differences in mating calls or visual displays in animals (e.g. Littlejohn *et al.* 1971, Gwynne & Morris 1986, Shaw *et al.* 1986, Baker & Baker 1990, Bendix & Howard 1991), or in plants, the separation of flowering times or

the utilization of species-specific pollinators. Post-mating/pre-establishment isolation includes prevention of fertilization and prevention of mitotic division because of unequal chromosome numbers (see Grant 1981) and embryo abortion. Post-establishment isolation occurs when unsuitability to the environment and hybrid sterility prevent further gene flow (see Barton & Hewitt 1985 and Hewitt 1989 for reviews).

If interspecific reproduction is successful, and interspecific gene flow is constant, hybrids will be produced, and will form a hybrid zone in association with the parental types. However, the long term outcome of hybridization will vary according to the circumstances. Continued hybridization between the parental species may suggest that the distribution of the hybrids will continually expand. However, Bigelow (1965) suggested that hybridization does not always result in progressively more similar gene pools. Hybrid sterility can cause the hybrid zone to become a "hybrid sink", because, as the population density within the hybrid zone will be continually depleted through selection against hybrids, there will be a higher level of migration from the parental gene pools into the hybrid zone, in order to maintain a population density equilibrium (Barton 1980). This hybrid sink may act to narrow the cline, or could result in a stable hybrid zone, if there is an equilibrium between hybrid selection and migration within the hybrid zone. Thus, the nature of the hybrid zone is a complex function of interspecific gene flow, hybrid fitness, environmental heterogeneity and historical factors.

Hybridization has long thought to have played a crucial role in evolution (particularly in plants) (Anderson & Stebbins 1954; Stebbins 1959; Kruckeburg 1969). However, although there are a few recent studies that have demonstrated a hybrid origin for some species (Rieseberg *et al.* 1988, Rieseberg *et al.* 1990, Arnold *et al.* 1990), the actual role of hybridization in reproductive isolation, and therefore speciation, is contentious (Arnold 1992). There are two main schools of thought that attempt to explain speciation as a result of the development of specific mating recognition systems: reinforcement and reproductive character displacement. The reinforcement model assumes that populations

become genetically differentiated in allopatry, and later come into secondary contact. If sufficient differentiation has occurred, the hybrids formed as a result of interpopulation mating are likely to be less fit than progeny produced by within-population matings (Dobzhansky 1951). This situation will create a series of clines for the loci at which the two species differ, and the widths of these clines will depend on the dispersal distance and the intensity of selection against the hybrids (Barton and Hewitt 1985). As a result of this selection, genes that increase the probability of assortative mating will be favoured, with the two populations subsequently forming two reproductively isolated species.

The character displacement model shares many elements of the reinforcement model, in that it assumes that gene pools will diverge in allopatry, with the resultant populations becoming reproductively isolated. However, if the two newly diverged populations come into contact, the most likely outcome is that the rarer population or allele combination will become extinct, rather than reinforcing the evolved differences (Paterson 1978). Butlin (1989) also argued that the effect of reinforcement is overridden by the recombination of genotypes within, and the continuous gene flow into the hybrid zone, and the eventual stabilization of selection on the mate recognition system. Both theoretical models (Paterson 1978, Sanderson 1989) and the apparent lack of unequivocal empirical evidence (Butlin 1989) suggest that speciation by reinforcement is unlikely. Despite the lack of support, however, reinforcement through hybrid disadvantage is a situation that may occur and cannot be ignored.

1.1.4. Secondary contact or primary intergradation?

The type of hybrid zone under study, hybrid fitness and the likelihood of speciation through reinforcement is partially dependent on the origin of the hybrid zone. Most of Barton and Hewitt's work has been based on the production of hybrids after secondary contact, while Endler put forward the notion of parapatric speciation as an alternative origin of hybrids. These two modes of hybrid zone formation have specific implications for hybridization and subsequent speciation.

Most of the hybrid zones described in the literature are thought to have originated as a result of secondary intergradation (see reviews of well-studied hybrid zones by Endler [1977], Barton & Hewitt [1985], Hewitt [1988, 1989]). Secondary intergradation is the result of a number of well defined steps. A population expands its range and is divided into two by a physical barrier. The populations diverge during isolation. The hybrid zone forms when the two previously allopatric populations come into contact with the breakdown of geographic or adaptational barriers, as a result of climatic change (many clines were formed after species expansion following the last glacial [Hewitt 1989]) or by man-made disturbance (e.g. Anderson 1948). If there has been insufficient divergence, interbreeding of the types will result in the production of hybrid individuals, forming a steep cline.

Endler (1977) argued that genetic differentiation can occur in the absence of geographic isolation, and despite high levels of gene flow (Slatkin 1973; Endler 1977). A cline formed within a continuous population is usually a response to environmental variation. A well known study on *Agrostis* and *Anthxanthum* is a classic example of speciation through primary intergradation (McNeilly & Antonovics 1968).

In studying natural hybridization, hybrid zones formed as a result of secondary or primary intergradation are difficult to differentiate (Endler 1977), because they can produce the same sort of geographic patterns. Knowledge of the history of the zone goes some way to differentiating between clines formed by primary and secondary intergradation, although the determination of the current extent of gene flow and the selection regime acting on the hybrids within the zone can help determine both the past formation and the fate of the hybrid zone.

1.1.5. Cline formation and maintenance

Hewitt (1988, 1989) proposed that a cline can arise between two groups fixed for different alleles at the one locus as a result of one of six selection regimes:

1. homozygotes and heterozygotes are equally fit ("neutral diffusion"),
2. one homozygote is fitter than both the other genotypes ("advancing wave"),
3. heterozygotes are less fit than the homozygotes ("adaptive speciation" or "tension zone"),
4. frequency-dependent homozygote fitness,
5. homozygotes fitter in different environments (environmental cline), and
6. heterozygotes fitter in the zone than the homozygotes ("hybrid superiority").

These models are based on single-locus explanations of possible selection regimes, but they are applicable to natural hybrid zones, because if two species are measurably different, the hybrid will be heterozygous for many sets of parental genes.

It was previously believed that hybrid zones were ephemeral (Moore & Buchanan 1985). Short lived hybrid zones would be expected under the first, second and (under some circumstances) the third selection regimes outlined above.

Under neutral diffusion, the species and the hybrids would mate randomly within the population, and as there is no selection against any of the individuals produced, the hybrid zone would widen as the populations merge (Moore & Buchanan 1985). This type of hybridization is commonly referred to as "introgressive hybridization", which is defined (and used throughout this thesis) as the infiltration of one group's genome into another's through hybridization and repeated backcrossing (Anderson & Hubricht 1938; Anderson 1953; Heiser 1969). Hybridization can occur without introgression, but the essential first step of introgression is hybridization. Although there are many published instances of introgression occurring in natural populations (e.g. Hardin 1975, Hopper

1977a, Barrowclough 1980, Arnold *et al.* 1990, Nason *et al.* 1992), these studies do not provide conclusive evidence that the hybrid zones are disappearing. Introgression, in fact, may also occur within tension zones and areas of hybrid superiority.

The advancing wave selection regime, in which one parental genotype is more fit than either the hybrids or the other parental genotype, would again result in an ephemeral hybrid zone. Because this type of selection is characterized by the movement of the cline through the population, the advancing wave is difficult to detect unless there is historical data on the zone. Not surprisingly, therefore, there are only a few reported instances of this type of hybrid zone (e.g. Yang & Selander 1968, Woodruff & Gould 1987, Gill 1980).

The production of unfit intermediates, through maladaptation or sterility, has long been thought to be the outcome of interspecific hybridization (Darwin 1859), and indeed, many hybrid zones exhibit evidence of unfit hybrids (e.g. Thaeler 1968, Kocher & Sage 1986, Shaw *et al.* 1986, Szymura & Barton 1986). Although Hewitt (1988) considered that selection against hybrids will result solely in a stable cline, selection against hybrids can also result in a short-lived hybrid zone, depending on the extent of gene flow. If the amount of gene flow between species is reduced after an initially high level (which resulted in the development of the hybrid zone), the cline would become increasingly narrower as a result of the reinforcement of reproductive isolation through selection against hybrid progeny, and eventually disappear. The scenario where premating reproductive isolation is reinforced by natural selection is known as the "adaptive speciation" hypothesis (Moore & Buchanan 1985), and is synonymous to speciation by reinforcement.

The alternative situation to adaptive speciation is where a stable cline is formed as a result of the constant production of and selection against less-fit hybrids. These zones have been termed "tension zones" (Key 1968), and the selection regime is also known as the

"dynamic-equilibrium" hypothesis (Moore 1977). These tension zones will tend to minimise their width (Hewitt 1988), and can move due to fluctuations of population density, coming to rest when the zone comes up against a barrier to gene flow (Barton & Hewitt 1989). A tension zone can also be formed by a frequency dependent selection regime (e.g. Mallet 1986), but there is a difference in the stage at which selection occurs (Hewitt 1988). These tension zones can be independent of environmental change, as they are formed solely due to internal genetic incompatibilities (Hewitt 1989).

The final two selection regimes require the selective superiority of genotypes in a particular environment, an essential pre-requisite of primary intergradation. This could involve the establishment of a particular genotype from an ancestral gene pool within a species resulting in a type of "founder effect", in which a stable or ephemeral hybrid zone (depending on the hybrid fitness) forms between the adjacent groups occurring on the populations' boundary, through secondary intergradation. This situation is the basis for Endler's (1977) parapatric speciation model. Alternatively, the hybrids may be adapted to a narrow ecotonal area between the parental habitats, with the parental genotypes unfit in the ecotonal habitat (e.g. dePamphilis & Wyatt 1990). This situation has been dubbed the "bounded hybrid superiority" hypothesis (Moore & Buchanan 1985), which is essentially a special case of the gradient model (Endler 1973, 1977).

A natural cline can result from a combination of a number of the above selection regimes (Hewitt 1988). Differential selection can manifest in differences in clines of measurable characters, such as morphology, enzyme chromosomes and DNA (see Hewitt (1989) for a list of systems exhibiting non-coincident clines). As a result of the possibility of non-coincident clines within a zone, Barton (1983) emphasises the need for all characters to be assessed separately, as well as in combination.

Generally, most of the models listed above, and indeed, most studies on natural hybrid zones, can be divided into those where (i) hybrids are selectively disadvantaged ("tension

zone"), and (ii) the cline is associated with an environmental gradient ("environmental cline"). Therefore, to determine how a cline under study arose, the first essential step is to determine the relative fitness of the hybrids to the parent genotypes, and if these genotypes across the cline are associated with any obvious environmental transitions. The specific model explaining the formation of the cline under study can be decided upon as more information on it's characteristics is gathered.

This thesis deals with a putative hybrid complex within the Australian genus *Banksia*, formed by *Banksia robur* Cav. and *Banksia oblongifolia* Cav. As will be seen in the following, this *Banksia* hybrid zone provides the opportunity to compare the importance of the "environmental cline" model versus the "tension zone" model in explaining the formation and maintenance of a hybrid zone, because the above anecdotal evidence suggests that the zone possesses traits of both models. In addition, because the distribution of the species is disjunct (forming a mosaic hybrid zone), this *Banksia* hybrid zone provides a potential instance for speciation by reinforcement, as well as independent replication needed to test the generality of the conclusions.

1.2. *Banksia*

The genus *Banksia* L.f. belongs to the Proteaceae, a diverse family of woody angiosperms with a strictly Gondwanan distribution. Seventy-five species have been identified (Taylor & Hopper 1988), although there is constant revision of the taxonomy (George 1988).

1.2.1. *Banksia* breeding system

Many aspects of the breeding system of the *Banksia* genus provide the opportunity for interspecific hybridization.

The pollinators of *Banksia* species are birds and small mammals (Collins & Rebelo 1987, Goldingay *et al.* 1987, Carthew 1993a), which are not species-constant foragers,

providing the opportunity for interspecific pollen to be transferred within the foraging range of these animals. *Banksia* flowers are arranged into a spike, numbering from a few to more than one thousand hermaphroditic flowers, which are thought to be protandrous (proposed by Carolin 1961, and confirmed by results for *B. coccinea* and *B. menziesii* in Fuss and Sedgley 1991a and b, respectively). Protrandry is highly correlated with high outcrossing rate (Holtsford & Ellstrand 1992). This tendency for protandry, therefore, reduces the likelihood of self pollination, and may provide the opportunity for interspecific pollen, deposited by inconstant pollinators, to fertilize the flower. Indeed, Lewis and Bell (1981) report the growth of pollen tubes of several species of *Banksia* in the stylar tissue of different species.

1.2.2. Hybridization in *Banksia*

There are only a few reported cases of hybridization in the genus *Banksia*, and most of these are between species in eastern Australia (George 1987), although many new presumed hybrids have been reported in a recent national survey of the genus (Taylor & Hopper 1988). Cultivated hybrid varieties are currently being developed, but until now, only one variety has gained commercial popularity: the hybrid between *B. ericifolia* and *B. spinulosa* var. *spinulosa* (known as *Banksia* "Giant Candles"). Natural occurrences of this hybrid, however, appear relatively rare (Henderson 1991).

The most commonly reported natural hybrid within the genus *Banksia* is that between *Banksia robur* Cav.and *Banksia oblongifolia* Cav., which has been recorded on many occasions where the two species co-occur. Hybrids between these two species may have been recognized as early as 1793, since a specimen collected by Luis Née in the Sydney region was in fact a hybrid (George 1987). Née who also collected the type specimens of *B. robur* and *B. oblongifolia* at the same time

Both *Banksia robur* and *B. oblongifolia* occur along 1500km of coast-line in eastern Australia, between Wollongong in the south and Rockhampton in the north (with isolated

3 0009 03132057 0

populations of *B. robur* occurring further north to Cooktown). The populations of *B. robur* are restricted to swamps and along creek and watercourses. These swamps and watercourses are often surrounded by woodland (Keith & Myerscough 1993), Populations of *B. robur* are therefore restricted to discontinuous pockets throughout its range. *Banksia oblongifolia* occurs in better drained regions, within *Eucalyptus* woodlands which often surround the *B. robur* swamps. An ecotone containing intermediates between the two species occurs in a narrow ring around the swamps (pers. obs.).

There are some obvious morphological differences between the *Banksia robur* and *B. oblongifolia*. *B. robur* leaves are very large (12-30 cm long, 5-17 cm wide), and highly xeromorphic. The inflorescences are variable in size, and are a striking metallic green in colour when the perianth is intact (Plate 1.1). *B. oblongifolia* leaves are much smaller (5-8 cm long and 1.5-2 cm wide), while their perianths are grey-yellow in colour (Plate 1.2). There is variation within this genus in morphology and habit: the variety showed in Plate 1.2, and the variety worked on in this thesis was given the variety name *oblongifolia* by Conran and Clifford (1987). They also identified a taller variety, with longer leaves, smaller number of basal stems and larger seeds (var. *minor*), although there is no evidence that there are genetic differences between the two varieties. The putative hybrids exhibit intermediate leaf size and perianth colour (Plate 1.3).

There has been little formal study (see Turner 1976, Elphinstone 1980) and no published work on this system. There may be several reasons for this: the hybrid does not increase the horticultural appeal of either species, and therefore does not represent a profitable avenue of study for horticulturalists. In addition, detailed information on the breeding system of *Banksia* species is only just starting to build (Scott 1980, Lewis & Bell 1981, Collins & Spice 1986, Whelan & Goldingay 1986, Carthew *et al.* 1988, Vaughton 1988, Fuss & Sedgley 1991a & b, Coates & Sokolowski 1992), and therefore, study within the genus, so far, has been largely restricted to within-species interactions. The genus is also

Plate 1.1. The *Banksia robur* inflorescence. The *Banksia* inflorescence consists of thousands of flowers arranged in pairs along a spike. In *B. robur*, flowers open sequentially from bottom to top (basipetally), but different portion may have differing rates of opening. The flowers of the lower portion of the inflorescence, shown here, have opened, but have yet to be foraged upon, as intact pollen bundles are visible as tan-coloured spheres on the tip of the style. The upper portion of the inflorescence show unopened flowers, with the striking metallic green perianth still intact over the pollen bundles. The size and shape of *B. robur*'s leaf is also evident in this picture. The leaf is longer and as wide as the inflorescence, and tapers into the petiole.

Plate 1.2. The *Banksia oblongifolia* inflorescence. The perianth is grey-yellow in colour in bud (on the right of the inflorescence), while the styles (on the left-hand side of the inflorescence) are yellow. It is also evident that the leaf is generally smaller than the those of *B. robur*.

Plate 1.3. The inflorescence of a presumed hybrid. The perianth colour and leaf size are intermediate between those of *B. robur* and *B. oblongifolia*.

difficult to work with, because of the plant's longevity and the difficulty of obtaining results from hand pollinations.

The previous studies on the *B. robur/B. oblongifolia* complex (Turner 1976 and Elphinstone 1980) came to the conclusion that the two species and their hybrids were part of the one "biological species". This conclusion was based on limited electrophoretic work, and the assessment that seed and pollen produced by putative hybrids were as viable as the parents'. If this conclusion is correct, the large variation in morphology suggests that there is huge phenotypic variation within this "species", associated with environmental variation. One of the aims of this thesis will be to determine if the "one biological species" hypothesis is correct, or that the morphological intermediates are indeed hybrids between two well defined species.

To determine if the intermediates are indeed hybrids of *B. robur* and *B. oblongifolia*, the criteria recognizing hybridization in natural populations, outlined in Section 1.1.2. of this Chapter, will be attempted in this study. Morphological and isozyme differences between the species and the putative hybrids will be documented, and confirmation of the occurrence of natural hybridization will then be attempted through controlled hand pollinations. *Banksia* is ostensibly an ideal system in which to perform controlled pollination experiments: pollen is generally readily accessible from the pollen presenters during the flowering season (see George 1987 for anatomy), and the inflorescence can be enclosed in mesh to prevent pollen removal by natural pollinators. Previous studies attempting hand pollinations in *Banksia*, however, have had generally low seed production (Carthew 1991 and Goldingay *et al.* 1991). In addition, because *Banksia robur* and *B. oblongifolia* are slow growing, comparison of artificially produced hybrids to putative natural hybrids has to be restricted to isozymes, because the production of reproductive hybrid plants could take decades. Observation of potential pollinators is relatively easy. Success in the tracking of mammals and direct observation of birds has been recorded (Paton & Turner 1978; Ford *et al.* 1979; Hopper 1980; Whelan &

Burbidge 1980; Goldingay *et al.* 1987; Ramsey 1989; Carthew 1993a; Vaughton 1992), and is facilitated by the accessibility of the large, showy inflorescences.

The close association of the species with the environmental conditions available has created a discontinuous hybrid zone along the east coast of Australia, an unusual situation among hybrid zones presented in the literature (see Endler [1977], Barton & Hewitt [1985] and Hewitt [1989] for reviews of published hybrid zone studies). Harrison (1986) has described a similar discontinuous distribution for a grasshopper in the United States, and has dubbed this type of hybrid zone a "mosaic hybrid" zone. Mosaic hybrid zones provide unique opportunities in the study of hybridization: the replication of observations on the species and hybrids in more than one independent interaction, and the study of perhaps one of the only situations conducive to isolation by reinforcement (Butlin 1989, Harrison & Rand 1989)

While the intermediates between *Banksia robur* and *B. oblongifolia* are common and widely recognized, they are less common than the parental species within the putative hybrid zones. Apart from external constraints (such as pollinator behaviour), one explanation may be that hybrids are less fit than the parental species. Alternatively, there are individuals within the hybrid zones that are morphologically intermediate between the presumed F_1 hybrids and parental plants, indicating that there may be extensive introgression, and therefore that the hybrid zone will be short-lived.

This *Banksia* hybrid complex, therefore, provides the opportunity to contribute to the many theoretical issues outlined in Section 1.1. The *B.robur/B. oblongifolia* complex shows characteristics that can be attributed to more than one of the hypotheses (Table 1.1), and the evidence supporting or disputing each of these will be addressed in this thesis.

Table 1.1. The issues pertaining to hybridization and hybrid zones being addressed in this thesis using the hybrid zone formed by *Banksia robur* and *B. oblongifolia*. Each issue has two or more associated hypotheses that can be specifically addressed.The (mostly anecdotal) evidence within the *Banksia* hybrid zone supporting each of the hypotheses has been listed. This evidence will be addressed more thoroughly within this thesis. The Models under "Cline formation" correspond to the listing on page 6.

	Issue	Hypothesis	Evidence of hypotheses in this *Banksia* hybrid zone
1.	**Morphological variation**	*Hybridization of species*	Plants with intermediate morphology coexisting with parental morpho-types.
		Phenotypic plasticity?	Previous studies of Turner (1976) and Elphinstone (1980)
2.	**Role of hybridization in speciation**	*Reinforcement*	Mosaic hybrid zone
		Character displacement	No firm anecdotal evidence, although introgression may be occurring
3.	**Cline formation**	*Model 1*	Some evidence of backcrossing and introgression
		Model 2	Cannot be addressed- long term study is essential
		Model 3	Relative rarity of hybrids
		Model 4	Relative rarity of hybrids
		Model 5	Hybrid zone may be dictated by environment - parental species seem to be suited to particular environmental conditions
		Model 6	Hybrids found in ecotonal areas
4.	**Origin of hybrid zone**	*Parapatric speciation: Endler (1977)*	Zone may be environmentally dictated
		Allopatric speciation: Barton & Hewitt (1981, 1985)	Disjunct distribution of species - unlikely to arise more than once?

1.3. The sites

The work described in this thesis was conducted in several sites within the Metropolitan Water Board Catchment area between Sydney and Wollongong. Two sites, containing mixed stands of *Banksia robur*, *B. oblongifolia* and intermediates, were located within the Cataract Catchment (150°51'20"E, 34°18'10"S) (referred to as the "Cataract" site), and the other within the O'Hare's Creek Catchment at Darkes Forest (150°54'45"E, 34°15'10"S) (the "Darkes Forest" site) (Figure 1.1). These sites were primarily sedge-heathland, permanently or intermittently waterlogged, surrounded by open eucalypt woodland. The Cataract site was part of a large swamp, approximately 1.6 km long and 500 m at the widest point, with the sample site covering an area of about 0.7 ha. This swamp had a running creek bisecting its length. The portion of the swamp used for sampling sloped towards this creek at an incline of about 1 in 300, with a south-east aspect (Plate 1.4). The 1.4 ha Darkes Forest site was part of a much smaller swamp (0.6 km long and 200 m wide). This area was different to the Cataract swamp in that it had no permanent running creek, although aerial photographs revealed a water course through the centre of the heathland. The sampling site had an almost imperceptible slope with a northern aspect (Plate 1.5).

Two populations of *B. oblongifolia* separated from stands of *B. robur* by at least 1 km, were selected within the Cataract Catchment and Darkes Forest (Figure 1.1.). Isolated populations of *B. robur* were more difficult to find, as the swamps in which they occur were always surrounded by woodland which contained *B. oblongifolia*. It was therefore necessary to find stands of *B. robur* that were large enough so that pollen exchange with the closest *B. oblongifolia* would be at a minimum. Three such stands were used: one near the Cataract hybrid zone site; one near Sublime Point Lookout (150°55'30"E and 34°17'32"S.); the third was located on the Picton Road, within the Cordeaux Catchment (Figure 1.1.). Each of the above stands will be referred to as "pure", "allopatric" or "isolated" populations of *Banksia oblongifolia* and *B. robur*.

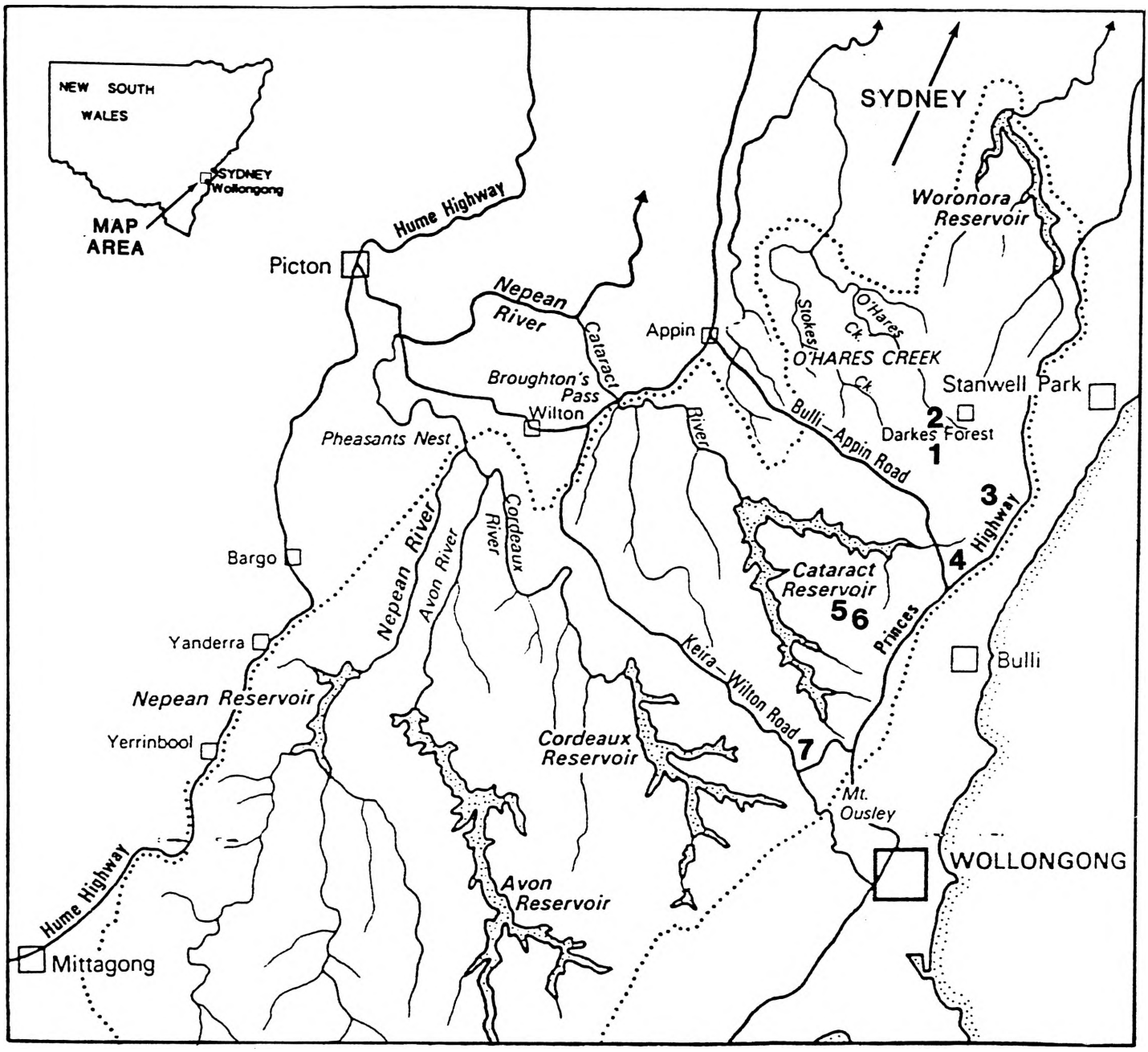


Figure 1.1. A map of the Water Board Catchment between Sydney and Wollongong. The six sites referred to within this thesis are marked: 1. Darkes Forest hybrid zone; 2. Darkes Forest pure *B. oblongifolia* site; 3. the burnt site on Madden's Plain; 4. Sublime Point pure *B. robur* site; 5. Cataract hybrid zone; 6. Cataract pure *B. oblongifolia* site; 7. Picton Road pure stand *B. robur*.

Plate 1.4. The Cataract hybrid zone site. Examples of *Banksia robur* (R) and *B. oblongifolia* (O) can be seen in the foreground. The swamp extends to the distant *Eucalyptus* stand, about 1km away. The creek running through the site can be seen in the centre of the swamp (indicate by arrow).

Plate 1.5. The Darkes Forest hybrid zone. This site is much smaller than the Cataract site. The *B. robur* (R) plants can be seen associated with sedges, which indicate the water course running through the site. The water level of this watercourse is not often above ground level.

One large quadrat was used to sample each site, because it was considered that other sampling methods such as transects and smaller quadrats over a larger area would not be adequate to detect the relationship of individuals within an area to one another, nor would they reflect the structure of the hybrid zone as a whole. The areas were defined by existing man-made and natural boundaries, such as fire trails, seismic lines, *Eucalyptus* forest or creeks. All plants within the area in each site were individually numbered.

1.4. Thesis aims

The general aims of this thesis are divided into three: 1. To describe the "parental species", hybrids, and the hybrid zones as they are at present (Chapters Two and Three). 2. To determine if hybrids are selected against at any stage of development (Chapters Three, Five and Six). 3. To determine the present potential for hybrid production, through the determination of the extent of reproductive isolation within the populations, preventing interspecific gene flow (Chapters Four, Five and Six).

Chapter Two

Morphological and genetic characterization of *B. robur*, *B. oblongifolia* and their putative hybrids.

2.1. Introduction

2.1.1. Morphological versus genetic detection of hybridization

The majority of hybrid zones are recognized because of the morphological or behavioural intermediacy of the organisms (see summary in Hewitt 1989). It is often difficult to classify an individual into a particular group using single characters because of the possibility of overlap between groups in the magnitude of the character. More sophisticated statistical methods, such as multivariate analyses (Adams 1982), have enabled the simultaneous use of all the measured characters to derive groups of organisms with similar quantitative and qualitative characters. The morphology of an individual may, however, be affected by environmental factors, and so observed variation need not have a genetic basis. Even if it does, introgression may obscure the interpretation of the morphological pattern of a group of organisms.

The occurrence of phenotypic plasticity has made it essential to gather independent genetic data (such as isozyme and/or DNA analysis, and henceforth in this thesis referred to, for simplicity, "genetic") to assess the extent of hybridization. The development of a variety of genetic techniques has made this possible, making it easier to classify individuals from presumed hybrid zones as parent species or F_1 and later generation hybrids, because the inheritance of genetic markers is usually much simpler than the inheritance of morphological and behavioural characters (Nason *et al.* 1992). The correlation of the instantaneously recognizable morphological characters with the more objective genetic markers is a sound way to characterize the organisms within a hybrid complex.

2.1.2. The *B. robur / B. oblongifolia* hybrid complex

2.1.2.1. Previous studies

Hybridization between *B. robur* and *B. oblongifolia* has been looked at in separate, unpublished studies by Turner (1976) and Elphinstone (1980). Turner looked at the morphology and phytochemistry of the two species and the presumed hybrid. As the study was short term, only leaf morphology was measured. Phytochemicals were extracted from the leaves. Creation of a morphology-based hybrid index indicated that the two species occupied the extremes of the range of the index, while the presumed hybrids were intermediate. Analysis of leaf phytochemistry revealed that some of the phenolic compounds isolated were present exclusively in each of the species. A combination of these parental compounds were often found within the putative hybrids, although many others were unique to the intermediates.

The study of *B. robur/B.oblongifolia* hybrids was extended by Elphinstone (1980), who looked at morphological characters, concurring with Turner (1976) on the morphological intermediacy of some plants. In addition, Elphinstone (1980) assessed electrophoretic variability and compared the viability of seeds and pollen of parental types and putative hybrids. She found no electrophoretic variation within the two species, nor were the two species fixed for different alleles. This lack of genetic variation, along with equality in pollen and seed viability of the two species and the presumed hybrids, prompted Elphinstone (1980) to suggest that *B. robur*, *B. oblongifolia* and their intermediates were part of the one biological species. Although attempting to characterize the morphs genetically was the appropriate approach, Elphinstone (1980) assayed only two enzymes and a general protein stain in screening for electrophoretic variation. It was, therefore, inappropriate and premature to conclude that these two species of *Banksia* were actually one species, based on so little data. Indeed, genetic surveys in other species of *Banksia* have shown that genetic variation is low (Scott 1980, Carthew *et al.* 1988), indicating that comprehensive screening may be required to find limited polymorphic loci, or even sufficient interspecific variation.

The lack of an objective method of classifying plants into parental versus hybrid groups is an obvious problem within these early studies. If *a priori* groupings of *B. robur*, *B. oblongifolia* and hybrids are based solely on morphological features, a hybrid index based on morphology will inevitably group them neatly into *B. robur*, *B. oblongifolia* and intermediate, but these groupings may not necessarily reflect the patterns of inheritance that produced them. As an illustration, if there are high levels of gene flow between *B. robur* and *B. oblongifolia*, and between the two species and the hybrids, individuals that may have been produced as a result of a hybrid/*B. robur* cross, may physically resemble *B. robur*, and may therefore be lumped with the *B. robur* group. The categorisation of a group based on genetic markers enables even the detection of the more complex patterns of introgression. Groupings based on genetic markers would therefore require a number of loci with clear differences between the species, and then an integration of the genotype and morphology. The more loci with fixed differences detected, the better defined will be groupings.

In this chapter, electrophoretic and morphometric data are used to characterize the pure stands of *B. oblongifolia* and *B. robur*. Using the genotypic and morphological limits set by these "pure populations", plants from sympatric populations of *B. robur* and *B. oblongifolia* are characterized genetically and morphologically, through the construction of separate genetic and morphological hybrid indices. Groupings within the sympatric populations and useful diagnostic characters are determined using multivariate analyses (Discriminant Function Analysis and Principle Components Analysis).

2.2. Methods

2.2.1. Determination of plant genotype

Electrophoresis was performed using an array of tissues: seed at various stages of germination including dry seed, early imbibition and early seedling stage up to full expansion of the cotyledons, as well as pollen and leaf material. Only seeds at the dry and

early imbibition stages produced consistently and reliably scorable zymograms, and the clarity of the allozyme bands did not vary between these two types of extracts. The maternal genotypes were thus inferred from the genotypes of arrays of seed progeny by the maximum likelihood method (Brown *et al.* 1975). Indeed, the determination of the genotype of the maternal plant from the genotype of its seed is more desirable in certain circumstances, because many alleles are expressed only at certain stages of development, and thus genotypes between different types of tissue from a plant may not be comparable (Brown & Allard 1970). This certainly seemed to be the case on the few occasions pollen used in electrophoresis yielded some scorable banding. ADH was the only enzyme in which banding was resolvable using pollen, but the number of loci and the mobility and number of alleles were different for pollen and seed, making direct comparison between the tissues impossible.

2.2.1.1. Screening for enzyme polymorphisms

Seeds were extracted from the follicles of newly collected infructescences and were placed in water overnight to ease removal of the seed coat. The seed (minus the seed coat) was homogenized in several drops of a Tris HCl buffer, which contained 10% sucrose, mercaptoethanol and bromophenol blue. The extracts were absorbed onto paper wicks, and these were inserted into the cathodal end of 12% w/v starch gels. After the electric field was applied, horizontal slices were then stained, using procedures modified from Selander *et al.* (1971), Harris and Hopkinson (1976) and Richardson *et al.* (1986).

Four to five seeds from the infructescences of around 10 randomly chosen plants from the pure stands were initially screened for activity and polymorphism using a number of enzymes. These enzymes, the buffer system used in running the gels and the number of loci and alleles resolved are listed in Appendix 2.1. The locus migrating furthest towards the anode was designated locus 1, the next furthest, locus 2, etc. Within each locus, the band with the fastest mobility was designated allele f, the next fastest, allele m, and the slowest was designated allele s.

Variation was resolved at four loci for *Banksia oblongifolia*: *Adh*, NSdh, *Sod* and *Gdh*. NSdh (Non-Specific Dehydrogenase) was apparently a non-specific dehydrogenase locus, since the same mobility and banding pattern was resolved on all dehydrogenases screened. Variation at this locus, however, was rare and dependent on the population assayed. Although no variation was detected at any locus resolved for *B. robur*, the species seemed to be fixed for an alternative allele to *B. oblongifolia* in *Adh*, *Sod* and *Gdh*, and fixed for the most common allele in *B. oblongifolia* at the NSdh. The *Adh* locus was monomeric, with only two alleles resolved. The remaining loci were dimeric, displaying one banded homozygotes and three banded heterozygotes. Three alleles for *Sod* and *Gdh*, and two alleles for the NSdh, were resolved.

2.2.1.2. Determination of maternal genotypes

Infructescences were collected from about twenty plants in each pure stand population. Infructescences were collected from a total of 136 plants from the Cataract and 90 plants from the Darkes Forest hybrid zones. The infructescences were burnt over a bunsen burner until the follicles were opened, submerged in water (because this enhances follicle reflexion) and left to dry. 15-20 seeds per plant were assayed, and the progeny genotypes were then used to determine the genotype of each maternal plant using the maximum likelihood method (Brown *et al.* 1975).

2.2.1.3. Genetic differentiation of plants

To obtain genetic information for classifying groups of plants, the frequencies of the alleles at *Adh*, *Sod* and *Gdh* in all the pure populations were determined, and these frequencies were used to devise a hybrid index. For each locus, the allele that was fixed for *B. robur* was given a score of 0. All other alleles were given a score of 1 (Table 2.1). This resulted in an individual score for each plant that ranged between 0 and 6 inclusive. A genetic hybrid index score (GHIS) was calculated for each of the plants that was able to be assayed within the hybrid zones.

Table 2.1. Scoring schedule for the construction of the genetic hybrid index, based on the genetic variation detected within the pure populations. For each plant, each allele detected at each locus was given a score of 0 or 1, and summed to obtain a genetic hybrid index score (GHIS). The minimum possible GHIS per plant is 0, while the maximum is 6.

Locus & Allele	Score*
Adh	
f	0
s	1
Sod	
f	1
m	1
s	0
Gdh	
f	0
m	1
s	1

* Each *B. robur* allele was scored as 0 and each *B. oblongifolia* allele was scored as 1.

2.2.2. Morphology

Each plant within the pure stands was characterized using its morphology. Leaf and inflorescence measurements were collected from each genotyped plant. Four mature leaves were used for each plant, one leaf from each branch orientated in the direction of the four points of the compass. One inflorescence from the previous flowering season that had not set seed (so as to have the smallest impact on future seed set) was also taken. The use of an inflorescence from the previous season was possible because the rigid rachises of *Banksia* can remain intact for many years, and the styles are persistent.

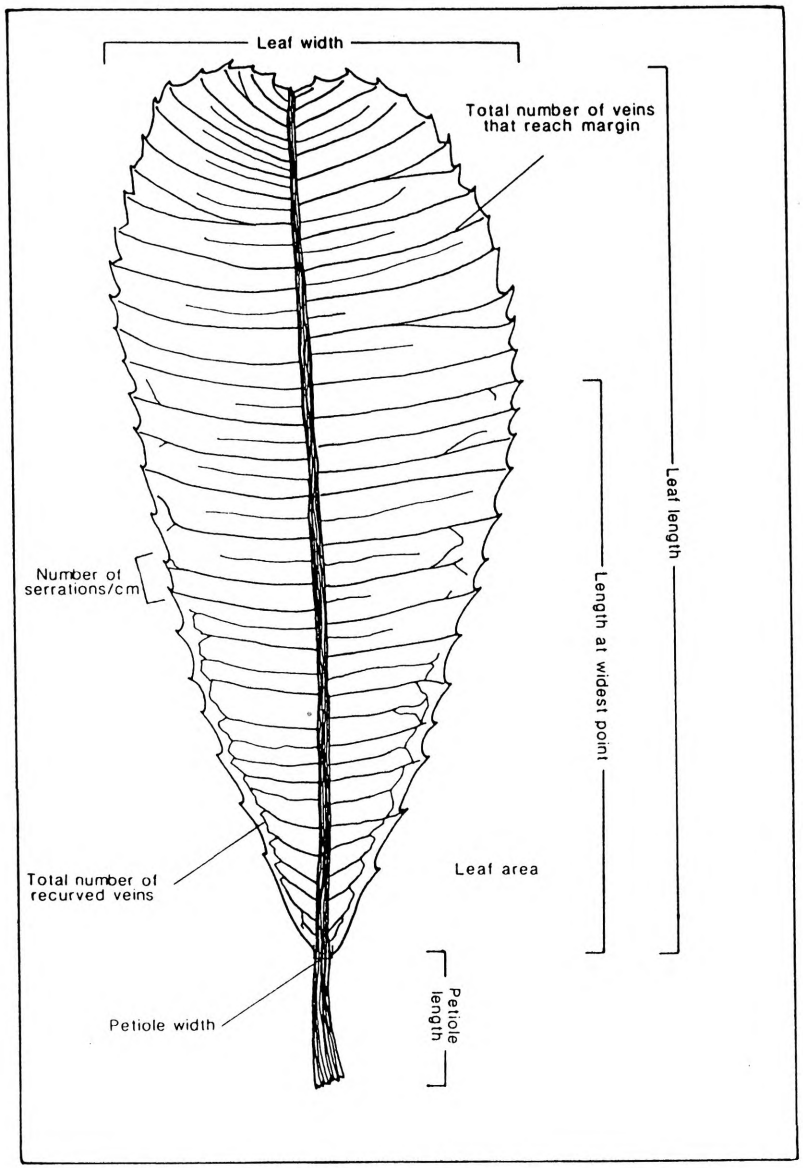
After collection all leaves were individually numbered, using random number tables, and then measured at random. This was to ensure that the measurements taken of a leaf were not biased by the measurements of other leaves taken from the same plant. Nine measurements were made on each leaf, and five measurements from each inflorescence (illustrated in Figure 2.1). The mean and standard error of each character were determined from the four leaves collected from each plant.

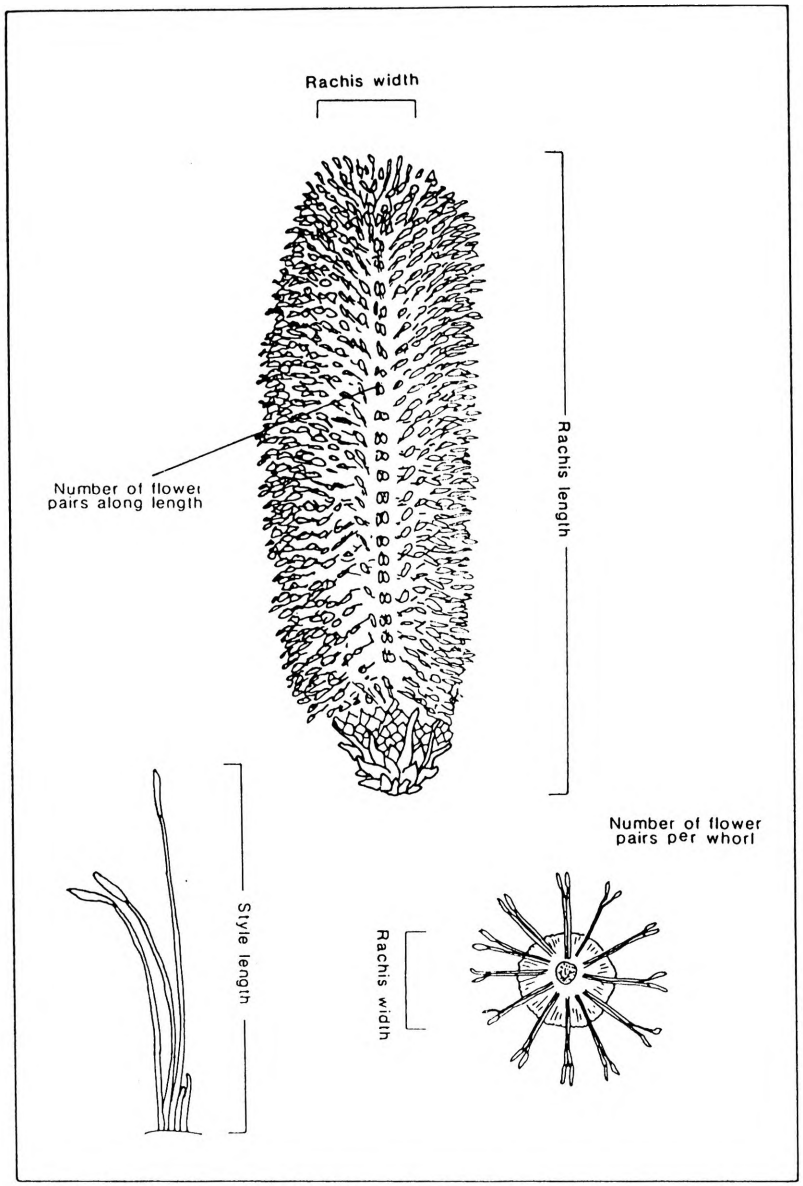
The same procedures were used for the collection of leaves and inflorescences from plants within the hybrid zones. In addition, a few fresh flowers, if available, were also collected from each plant to determine the colour of the perianth. The perianth colour was matched to one of an array of 15 colours (Figure 2.2) obtained using Dawes (1990), which produces colour samples the four-colour tint process.

2.2.2.1. Morphological predictions

From descriptions of the two species in George (1981, 1987), it was expected that measures of leaf size, such as leaf length, width and area, petiole length and width, are expected to separate *Banksia robur* and *B. oblongifolia* very well. There is a striking difference between species in the perianth or inflorescence colour (see Figure 2.2), so colour also seemed likely to be a useful separating character. According to the descriptions given in George (1987), the floral characters, such as inflorescence length

Figure 2.1. The morphological characters measured for each plant. a. illustrates the nine leaf characters measured. b. The floral characters measured from the inflorescence and individual flowers.





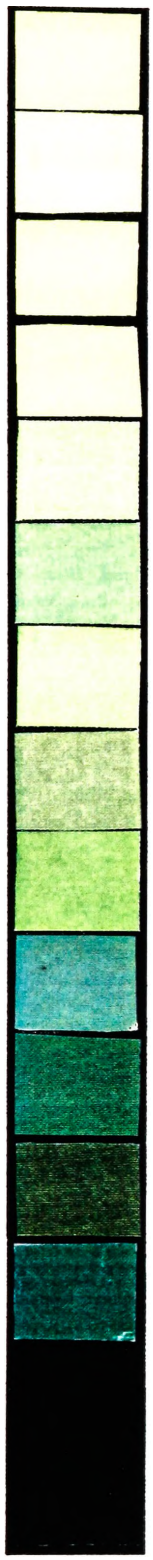


Figure 2.2. The array of colours used to identify the colour score of the perianth for each inflorescence collected. Each inflorescence was assigned a score between 1 and 15 inclusive. It was more practical in later exercises to divide the array into three colour intensities: light (scores of 1-5), medium (scores of 6-10) and deep (scores 0f 11-15).

and width, flower numbers and style length, overlap in size, and therefore were thought to be poor discriminators of these species.

Plate 2.1 illustrates the difference in venation used in this study: the *Banksia robur* leaf generally show veins that continue to the edge of the leaf margin, ending in a spine, while for *B. oblongifolia*, the major veins originating at the midrib curve back before reaching the leaf margin, resulting in fewer spines. *B. robur* is expected to have a higher proportion of marginal veins to recurved veins, while the reverse will be true for *B. oblongifolia.*

The last set of predictions was based on leaf shape, for which some of the leaf size characters were used. A plot of the length to widest point was used as the numerator in a ratio with the leaf length, against the ratio between leaf width and leaf length was used in an attempt to summarize any difference in leaf shape. The leaf shape for *B. robur* is described as being elliptical, and oblong in *B. oblongifolia* (George 1987). Therefore, for *B. robur*, the predicted magnitude of both the length to widest point/ length ratio and the width/length ratio would be approximately 0.5, while for *B. oblongifolia*, the length to widest point/ length ratio would be fairly small and the width/length ratio would be less than 0.5.

2.2.2.2. Morphology hybrid index

To affirm that the morphological measurements made on plants in pure stand *B. robur* and *B. oblongifolia* were characteristic of the species, the significance of the differences between the measurements taken from the plants in each of the pure stands was determined using a 1-way ANOVA (Model I, with plants with the hybrid index score of 0, 5 and 6 being the three levels within the fixed factor). A Tukey multiple comparisons test was performed on those characters that revealed significant differences between levels.

Plate 2.1. Comparison of the venation shown by the two species and the hybrid (*B. robur* on the left, and *B. oblongifolia* on the right, and the hybrid in the centre). The recurvature of the predominant veins are clear on the *B. oblongifolia* leaf (indicated by arrow), while on *B. robur*, the veins invariably end in a spine at the margin (indicated by arrow). The hybrid has examples of both recurved (r) and marginal (m) veins.

In constructing the morphological hybrid index, the characters that were not significantly different between the *B. robur* and *B. oblongifolia* groups, were discarded. The index was then constructed using the remaining characters, achieved by comparing the standard deviation of the measurements from the pure stand *B. oblongifolia* to those from the pure stand *B. robur*, in order to delineate *B. oblongifolia*, intermediate and *B. robur* regions along the size range of the character (according to Hopper 1977a) (Figure 2.3 illustrates how the cut off points for *B. oblongifolia*, intermediate and *B. robur* measurements were determined). Each region was assigned a score: 0 for *B. oblongifolia*, 1 for intermediate and 2 for *B. robur* (c.f. the genetic index - Table 2.1). The scores for each measurement were then added together to obtain a morphology hybrid index score (MHIS) for each plant. Any significant association between the morphological index score and the hybrid index score of the plants was tested using a contingency table.

To determine if there were significant differences between the means of each group of plants defined by each score on the genetic hybrid index, a Model I 1-way ANOVA was performed for each morphological character. The source of any significance detected was determined using a Tukey multiple comparisons test.

2.2.2.3. Discriminant Analysis

Discriminant analysis was used to determine the combination of characters that best discriminated between the seven genetic hybrid scores. This analysis calculates a linear function that is derived from the combination of independent variables that best discriminate between the *a priori* defined groups (Hair *et al.* 1987). Four separate analyses were performed. These consisted of different character combinations: leaf only characters, leaf plus colour, leaf and floral characters and total character array (measurements of leaf, floral and colour). The four different analyses were performed to determine if different character combinations better defined the groups when analysed with other characters (Hair *et al.* 1987), but the small sample size associated with the total character array made it difficult to rely solely on the discriminant function calculated from

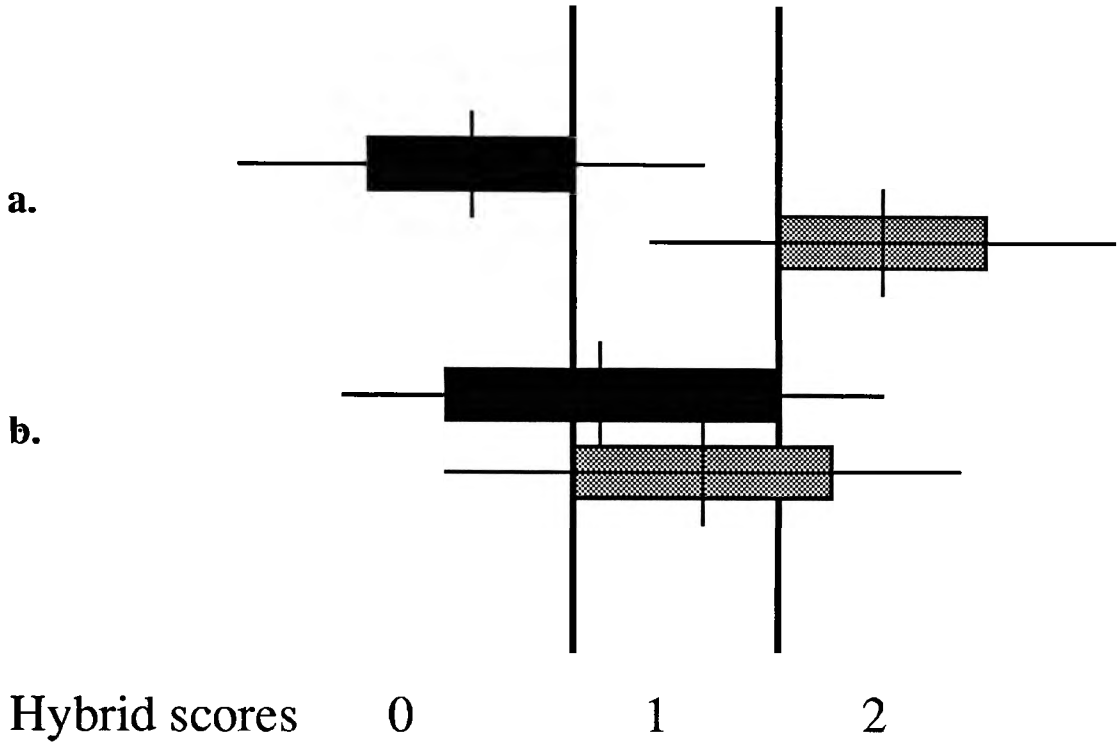


Figure 2.3. Illustration of the system for the allocation of scores for 2 hypothetical characters, a and b. The cut-off points were determined using the character measurements taken from the pure stand plants. The black bars represent the standard deviation of the measurements for a character for species 1, while the stippled bars represent the standard deviation of the measurements for a character for species 2. Horizontal lines represent the range of measurements, while the vertical line is the mean. The continuous vertical lines show the cut-off points for three different situations. The final MHIS for each plant was calculated as the sum of the individual scores for x characters. Modified from Hopper (1977a).

these data. For simplicity, the results obtained for the group of characters that best classify the plants into genetically defined groups only will be presented. The discriminant functions were computed using the SAS package.

For each character used in the analysis, loadings (sometimes known as the structure correlations) and the weights (known as the discriminant coefficients) indicate the relative discriminatory power of that character. A loading with a comparatively high absolute value indicates a high level of correlation between the character and the function, while the higher the absolute value of the weight, the better the character is at separating groups. Negative and positive loadings and weights indicate whether there is a negative or positive contribution to the function. Weights are probably most commonly used to indicate high correlation with the discriminant function, but should be interpreted with caution, as the effects of the character may be underestimated because of collinearity, as they are subject to instability (Hair *et al.* 1987). It is therefore safer to determine the structure of the function using the loadings, and therefore the interpretation in this study will concentrate on the loading values. An additional piece of information provided by the analysis is the classification matrix, which indicates the accuracy of the function at classifying plants into each GHIS.

2.2.2.4. Principal Component Analysis

Factor analysis was used to determine if distinct groups within the hybrid zones could be separated using the morphological characters measured. The most commonly used factor analysis, Principal Component Analysis (PCA), was chosen as the method for factor extraction, as a major concern was to predict the usefulness of the characters in grouping the individuals. This model uses the total variance within the data set, so a precondition in the use of component analysis is that the variances attributed to error within a specific character are negligible (Hair *et al.* 1987). The number of factors extracted was determined by a 75% variance criterion (Hofmann & Simpson 1986). The Orthotran Transformation solution was employed, using Statview 512+. This solution clarifies the

simple structure of an orthogonal rotation by allowing the factors to be correlated, so that the interpretation of the factors is as simple as in an oblique rotation (Hofmann & Simpson 1986).

PCA was performed using several separate combinations of the morphological characters measured. The character array with the largest sample size, leaf characters only, was used in the first analysis. Inflorescence data was combined with leaf characters for the second analysis. Perianth colour was added to the leaf character array in the next analysis, and the last consisted of the total possible character array (leaf characters, perianth colour and inflorescence measurements). The separate analyses were performed to determine if sample size and character combination had an effect on the groupings, as it was impossible to collect inflorescences from all individual plants to obtain floral size and colour characters. The results of all these analyses will be presented.

The factor scores, obtained for every plant after each analysis, were plotted so that the resultant groupings could be visualized. Three separate plots were obtained for the first and second analyses. The plants from the pure populations were plotted first to determine if the two species could be separated. Secondly, the factor scores obtained for the plants from the hybrid zones were plotted. The hybrid plants were separated from the hybrid zone group to determine if there were distinct groupings within these plants. The second and third plots were only plotted for the first two analyses, as the last two contained no plants from the pure stands.

2.3. Results

2.3.1. Genetic variation

There were fairly clear genetic differences between *Banksia robur* and *B. oblongifolia.* The pure populations of *Banksia robur* were fixed for one allele at each of the four loci that showed variation within the *B. oblongifolia* pure populations (Table 2.2). Only the *Adh* locus in the Cataract *B. oblongifolia* pure stand and the *Gdh* locus in the Darkes

Table 2.2. Frequency of alleles in four loci within the five pure populations surveyed (three *B. robur* and two *B. oblongifolia*). (n) is the number of plants from each population used in the calculation of the frequencies.

	B. robur			*B. oblongifolia*	
Locus Allele	**Cataract**	**Sublime Point**	**Picton Road**	**Cataract**	**Darkes Forest**
Adh					
f	1.00	1.00	1.00	0.0	0.07
s	0.00	0.00	0.00	1.00	0.93
NSdh					
f	1.00	1.00	1.00	1.00	0.98
s	0.00	0.00	0.00	0.00	0.02
Sod					
f	0.00	0.00	0.00	0.43	0.48
m	0.00	0.00	0.00	0.40	0.33
s	1.00	1.00	1.00	0.17	0.19
Gdh					
f	1.00	1.00	1.00	0.13	0.00
m	0.00	0.00	0.00	0.40	0.57
s	0.00	0.00	0.00	0.47	0.43
(n)	15	15	15	15	21

Forest *B. oblongifolia* pure stand showed unique alleles: on every other locus there was a small proportion of the "*B. robur* allele". Although the NSdh locus showed some variation within the *B. oblongifolia* populations, the alternative allele was rare.

All plants within the *B. robur* pure stands scored 0 on the genetic hybrid index. The presence in small frequencies of "*B. robur* alleles" within the *B. oblongifolia* pure populations resulted in these plants having GHISs of either 5 or 6, and not just the maximum of 6 that would have been expected. The pure stand *B. oblongifolia* plants, therefore, were roughly equally divided between GHISs of 5 and 6 (Figure 2.4)

The genetic survey of plants within the hybrid zones showed plants with a full range of GHISs along the genetic hybrid index. The highest proportion of plants in both populations scored 0, while only fairly small proportions scored 5 and 6 (Figure 2.5). In both populations there was a substantially smaller number of plants scoring 6 compared to those scoring 5.

There was a full range of genetically intermediate plants within both hybrid zone populations (Figure 2.5). These data confirm that hybridization is occurring between *B. robur* and *B. oblongifolia*, and suggest there is extensive introgression occurring within the hybrid zones. Of the intermediate plants, those with a GHIS of 3 represented the greater proportion (Darkes Forest 17.8%, Cataract 17%).

2.3.2. Morphology

The plants from pure stands with a GHIS of 0 (*B. robur*) were able to be adequately separated from pure stand plants with GHISs of 5 and 6 (*B. oblongifolia*), on the basis of many of the morphological characters measured, though some characters exhibited greater discriminatory power than others. The separation of plants which scored 0 from those scoring 5 and 6 in the hybrid zones using the same characters was not as definite, but was

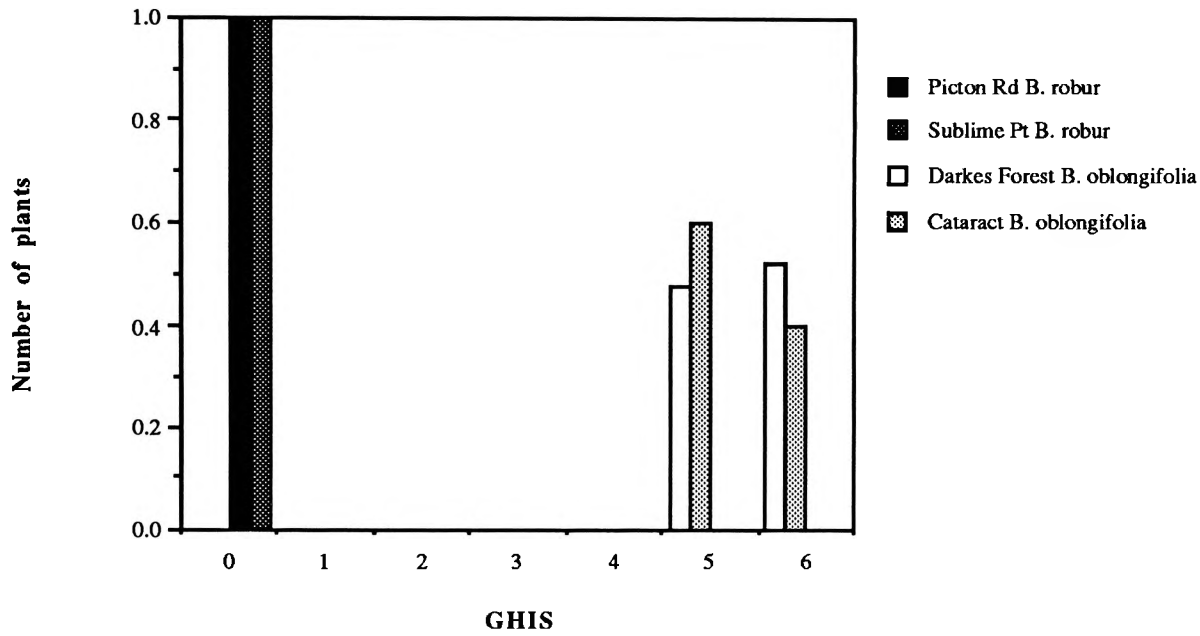


Figure 2.4. The proportion of each GHIS in the pure populations of *B. robur* and *B. oblongifolia*. Sample sizes are: Picton Road and Sublime Point *B. robur* and Cataract *B. oblongifolia*=15, Darkes Forest *B. oblongifolia* = 21.

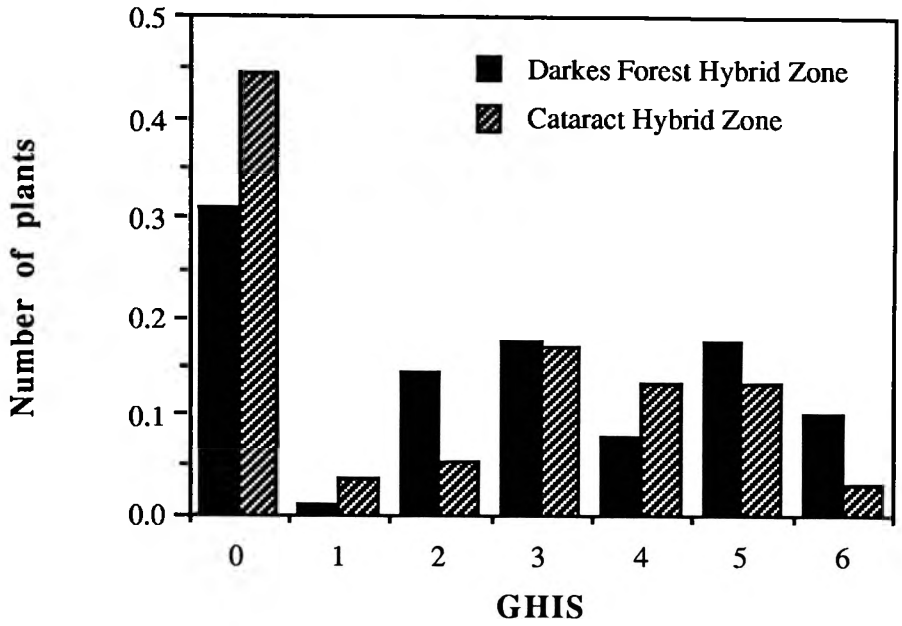


Figure 2.5. The proportion of each GHIS in the hybrid zone populations of Darkes Forest and Cataract. Sample sizes are: Darkes Forest=90, Cataract=136.

still adequate to group them. The hybrids formed a continuum between the parental types, with the suggestion that plants genetically closer to *B. robur* (i.e. with GHISs of 1 and 2) were morphologically more *B.robur* like, while those plants scoring 4 were morphologically similar to *B. oblongifolia.*

2.3.2.1. Pure Stand Morphology

The length to widest point was used as the numerator in a ratio with the leaf length, plotted against the ratio between leaf width and leaf length (Figure 2.6). Separation was evident only using the width/length ratio, where the predictions made in Section 2.2.2.1. were largely borne out by the results. However, the length to widest point/length ratio did not reveal any difference between the two species .

The plot of the number of recurved veins versus the number of veins extending to the leaf margin showed a complete separation of *B. robur* and *B. oblongifolia* (Figure 2.7).

Well defined separation of the pure stand plants in the sizes of the characters measured was found for leaf length, leaf width, leaf area, petiole length and petiole width (Figures 2.8a, b, c, d and e, respectively). This definition, however, was not apparent in either serrations per centimetre of leaf margin nor in any of the inflorescence characters. Specifically, the serrations per centimetre of leaf margin, rachis length and number of flower pairs along the length of the rachis showed no separation of the two species, while there was a large region of the size range of rachis width, number of flower pairs per whorl and style length where *B. robur* and *B. oblongifolia* overlapped.

This pattern was confirmed by the use of one factor ANOVAs using the GHI of 0, 5 and 6 within the pure stands as the levels within the factor (Appendix 2.2). Significant differences were detected between GHISs in 11 of the 14 characters (leaf length, leaf width, length from leaf base to widest point, leaf area, petiole length, petiole width, recurved veins, marginal veins, inflorescence width, pairs of flowers per whorl and style

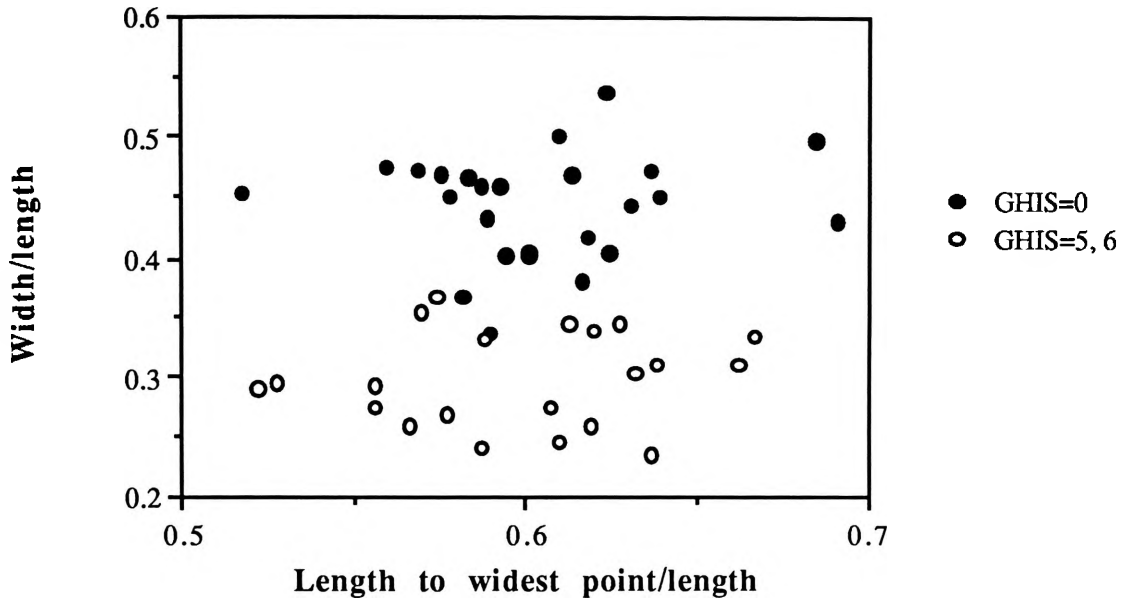


Figure 2.6. Plot of the ratio of leaf width to leaf length against the ratio of length to widest point to leaf length of plants from the pure stands only.

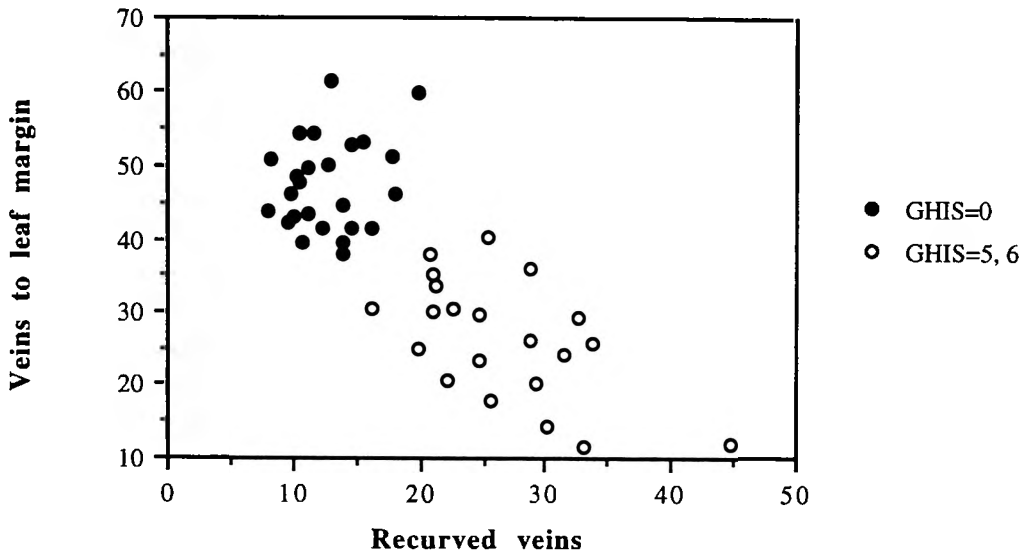


Figure 2.7. Plot of the number marginal veins against the number of recurved veins for plants from the pure stands.

Figure 2.8. Stack column graphs of the size classes for each character measured from plants found in the pure stands. a. leaf length, b. leaf width, c. leaf area, d. petiole length, e. petiole width, f. serrations per centimetre of leaf margin, g. rachis length, h. rachis width, i. number of rows of flower pairs along length of rachis, j. number of flower pairs per whorl, and k. style length. For each graph, black bars are the plants from the pure stand *B. robur*, white bars are pure stand *B. oblongifolia* with hybrid index scores of 5 and 6.

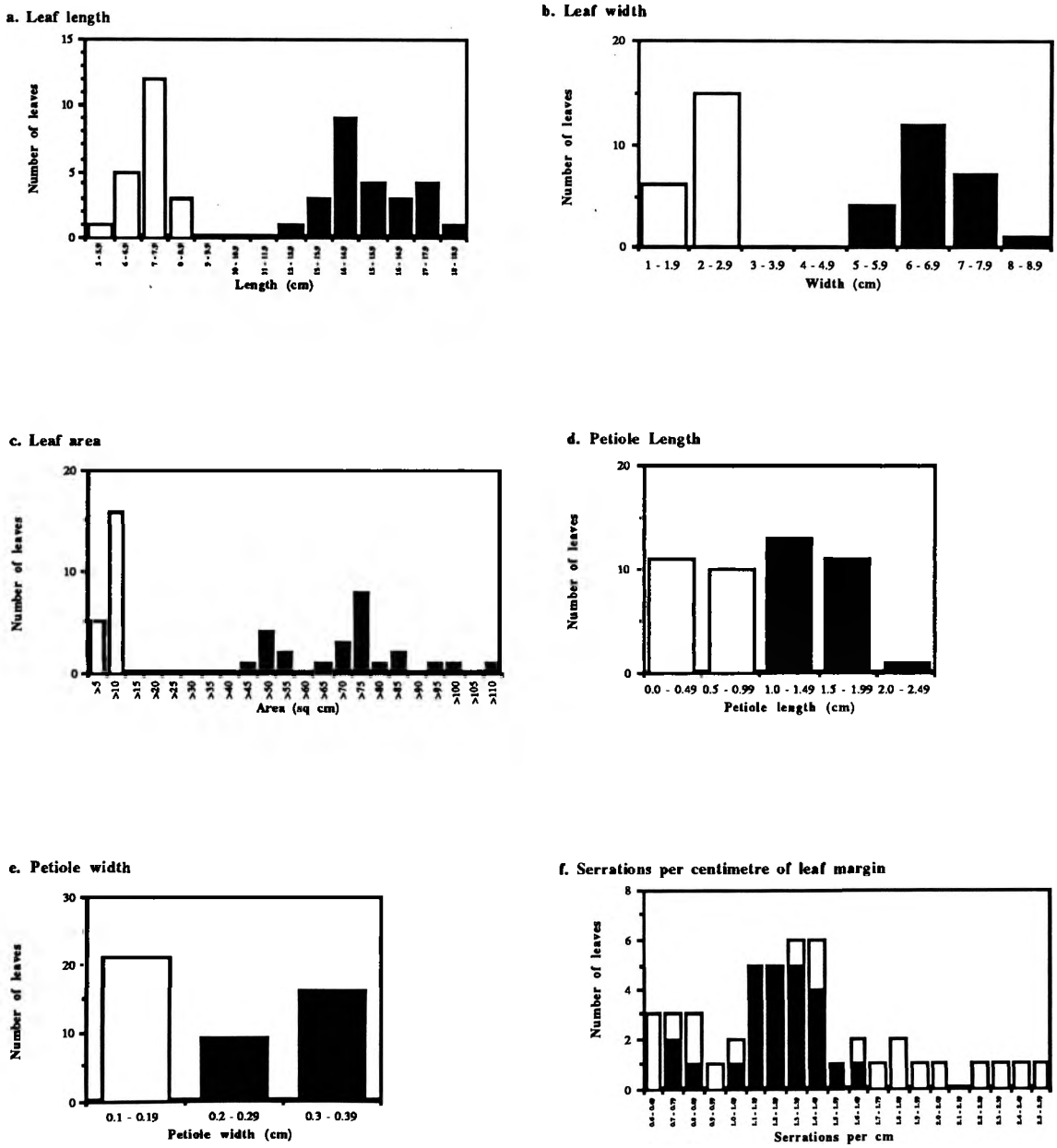
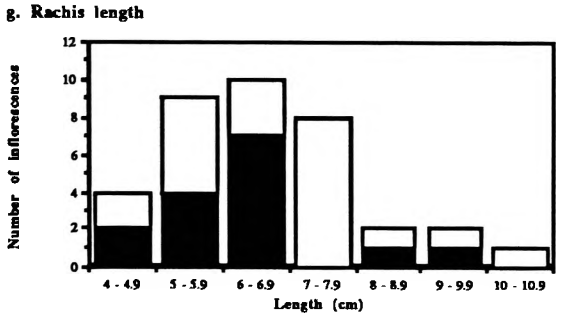
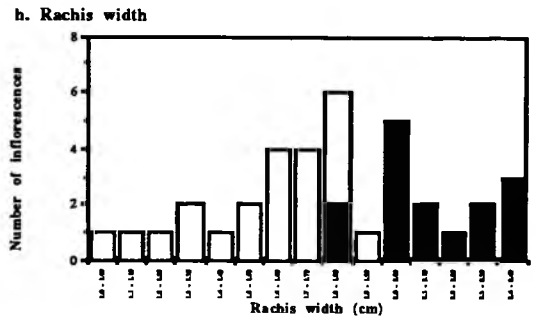
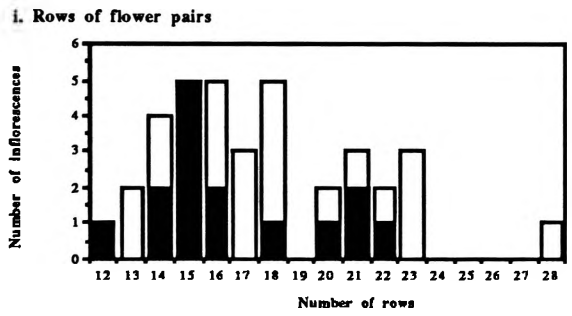
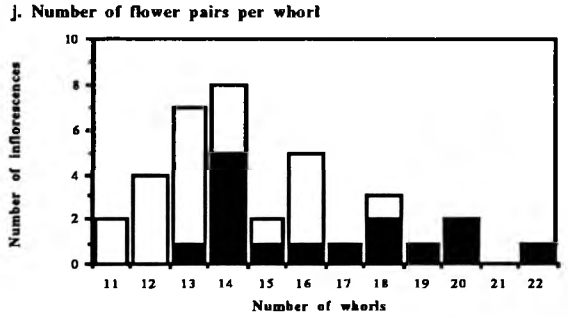


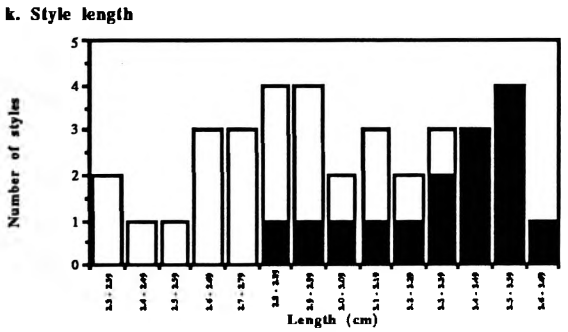
Figure 2.8 continued











length). In all of these measurements, a multiple comparisons test (Tukey) revealed that, although there were no differences between the plants with GHISs 5 and 6, these plants were significantly different from the plants from the *B. robur* stands (GHIS=0) (Table 2.3).

2.3.2.2. Morphology of Hybrid Zone Plants

Significant differences between the genetic hybrid index scores were detected for all characters using a 1-way ANOVA (Appendix 2.3). Multiple comparison testing (Tukey) revealed that in eight characters (leaf length, leaf width, length to widest point, leaf area, petiole length, petiole width, number of recurved veins and number of flower pairs per whorl), the group of plants with a GHIS of 0 were significantly different from all other GHISs (Table 2.4). In the number of marginal veins, rachis length and width, rows of flower pairs and style length, plants with GHIS=0 were not significantly different from one or more other scores. *Banksia oblongifolia* plants (GHIS=5 and 6) were always grouped with one or more other GHISs.

To simplify the graphical representations of the morphological characters measured, the plants within the hybrid zones classified using the genetic hybrid index were divided into three groups: all plants with a GHIS of 0 were grouped as "*B. robur*-type", plants with GHISs of five and six were grouped as "*B. oblongifolia*-type", the plants with GHISs of 1, 2, 3 and 4 were grouped together as hybrids.

The ratio of leaf width to length was plotted against the ratio of the length to the widest point to length separated the *B. robur*-type plants and the *B. oblongifolia*-type plants within the hybrid zone almost as well as for the plants from the pure stands (Figure 2.9). The majority of hybrid plants were closely associated with the *B. oblongifolia*-type plants.

Table 2.3. Morphological characters measured from plants collected in the pure stands. The figures are the means for each character within each hybrid index (with the standard error in parentheses). Hybrid index of zero are the plants from the pure stand *B. robur*, while hybrid indices of five and six are from pure stand *B. oblongifolia*. The black bars show the results of the Tukey test performed on the means (at $\alpha = 0.05$) based on the results of the one-way ANOVA (results presented in Appendix 2.2). The bars join the hybrid indices where no significant difference was detected Sample sizes are: hybrid index=zero - characters a-i, sample size=25; characters j-n, sample size=15; hybrid index=five - characters a-n, sample size=10; hybrid index=six - characters a-n, sample size=11.

	Character	Zero	Five	Six
a.	Leaf length	15.3 (0.31)	7.4 (0.26)	7.3 (0.13)
b.	Leaf width	6.4 (0.28)	2.2 (0.08)	2.2 (0.08)
c.	Leaf to widest point	10.0 (0.73)	4.5 (0.14)	4.3 (0.12)
d.	Petiole length	1.5 (0.06)	0.5 (0.03)	0.5 (0.25)
e.	Petiole width	0.3 (0.007)	0.2 (0.01)	0.2 (0.01)
f.	Serrations	1.2 (0.46)	1.5 (0.23)	1.44 (0.19)
g.	Recurved veins	12.7 (0.62)	25.5 (1.70)	27.6 (2.25)

Table 2.3. continued

	Character	Zero	Five	Six
h.	Marginal veins	47.3 (1.27)	28.1 (2.34)	24.7 (2.67)
i.	Leaf area	74.3 (3.38)	10.7 (0.66)	11.3 (0.40)
j.	Inflorescence length	6.3 (0.35)	7.1 (0.39)	6.7 (0.51)
k.	Inflorescence width	2.2 (0.05)	1.6 (0.08)	1.6 (0.07)
l.	Rows of flower pairs	16.6 (0.79)	18.4 (0.98)	18.3 (1.39)
m.	Flower pairs per whorl	16.4 (0.72)	13.3 (0.54)	14.0 (0.62)
n.	Style length	3.3 (0.06)	2.7 (0.07)	2.8 (0.10)

Table 2.4. Morphological characters measured from plants collected within the hybrid zones The figures are the means for each character within each hybrid index (with the standard error in parentheses). Within each character, hybrid indices are ordered in decreasing order of the mean. Hybrid index =1 is excluded from the inflorescence characters (characters j-n) as no inflorescence could be collected from any of these plants. The results of the Tukey test performed on the means (based on the ANOVA results presented in Appendix 2.3) are represented by the black bars beneath each character. The bars join the hybrid indices where no significant difference was detected. Sample sizes for characters a-i: HI=0, 44; HI=1, 4; HI=2, 14; HI=3, 27; HI=4, 17; HI=5, 25; HI=6, 8. For characters j-n: HI=0, 26; HI=2, 9; HI=3, 16; HI=4, 13; HI=5, 13; HI=6, 4.

a. Leaf length	**0**	**2**	**3**	**1**	**5**	**6**	**4**
	16.9	11.6	10.4	10.3	7.5	7.5	7.3
	(0.26)	(0.80)	(0.53)	(1.29)	(0.37)	(0.29)	(0.35)
b. Leaf width	**0**	**2**	**1**	**3**	**5**	**4**	**6**
	7.1	4.0	3.6	3.4	2.3	2.2	2.1
	(0.15)	(0.32)	(0.71)	(0.22)	(0.12)	(0.14)	(0.09)
c. Leaf to widest point	**1**	**2**	**1**	**3**	**6**	**4**	**5**
	10.1	6.9	6.5	6.4	4.6	4.6	4.6
	(0.23)	(0.51)	(0.68)	(0.35)	(0.19)	(0.21)	(0.19)
d. Petiole length	**0**	**2**	**1**	**3**	**5**	**6**	**4**
	1.6	0.9	0.8	0.7	0.5	0.5	0.4
	(0.04)	(0.07)	(0.12)	(0.04)	(0.03)	(0.03)	(0.03)
e. Petiole width	**0**	**1**	**2**	**3**	**6**	**4**	**5**
	0.33	0.22	0.22	0.20	0.16	0.15	0.15
	(0.007)	(0.033)	(0.012)	(0.010)	(0.007)	(0.004)	(0.004)
f. Serrations	**4**	**3**	**5**	**2**	**1**	**6**	**0**
	1.76	1.56	1.50	1.49	1.46	1.42	1.32
	(0.086)	(0.100)	(0.083)	(0.063)	(0.155)	(0.202)	(0.030)

Table 2.4. continued.

g. Recurved veins	6	5	4	1	2	3	0
	32.7	30.0	27.2	26.3	25.1	24.5	15.7
	(2.79)	(1.39)	(1.18)	(2.35)	(2.01)	(1.28)	(0.44)
h. Marginal veins	0	2	3	1	4	5	6
	48.5	37.4	35.0	33.4	28.2	24.3	24.1
	(1.50)	(2.45)	(2.76)	(4.12)	(2.33)	(1.61)	(3.80)
i. Leaf area	0	2	1	3	5	4	6
	88.7	35.8	29.1	27.2	12.4	12.0	10.9
	(3.15)	(5.07)	(7.71)	(3.06)	(1.49)	(1.44)	(0.62)
j. Inflorescence length	0	6	2	5	3	4	
	9.4	9.2	8.9	7.2	6.9	6.0	
	(0.45)	(2.12)	(0.86)	(0.58)	(0.52)	(0.06)	
k. Inflorescence width	0	2	6	3	5	4	
	2.4	2.1	1.7	1.7	1.7	1.5	
	(0.08)	(0.18)	(0.32)	(0.10)	(0.07)	(0.46)	
l. Rows of flower pairs	0	6	2	3	5	4	
	23.1	23.0	22.3	20.3	20.0	17.62	
	(0.87)	(3.76)	(1.93)	(1.22)	(1.05)	(0.89)	
m. Flower pairs per whorl	0	2	6	3	5	4	
	20.2	16.4	15.8	15.1	14.2	13.4	
	(0.70)	(0.18)	(1.49)	(0.64)	(0.70)	(0.05)	
n. Style length	2	0	6	3	5	4	
	3.18	3.10	3.05	2.91	2.87	2.71	
	(0.861)	(0.073)	(0.119)	(0.09)	(0.082)	(0.389)	

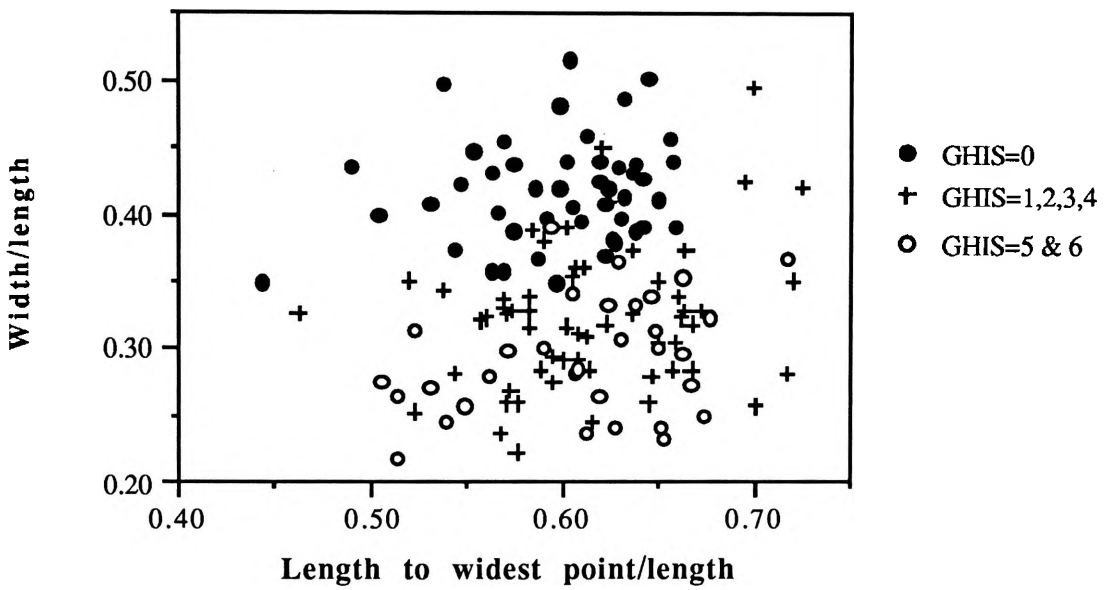


Figure 2.9. Plot of the ratio of leaf width to leaf length against the ratio of length to widest point to leaf length of plants from the hybrid zones.

The plot of the marginal veins against the recurved veins separated the two parental types found within the hybrid zones (Figure 2.10). As in Figure 2.8, the hybrid plants ranged over half the range of the *B. robur* type plants, and over the full range of the *B. oblongifolia* plants.

Within the hybrid zone, the *B. oblongifolia*-type group and *B. robur*-type group were completely separated in leaf length, width, area, petiole length (Figure 2.11 a, b, c and d), while there was only small overlap in rachis width and petiole width (Figure 2.11 e and h). However, the separation of many of the characters are not as well defined as the separation observed between the plants from the different pure stands. In many of the characters measured, the hybrid plants range extends through much of the range of both the *B. oblongifolia* and *B. robur* type plants.

In order to summarize the association between the genotype and morphology of the plants within the hybrid zone, a frequency histogram of each morphology hybrid index score (MHIS) was plotted. The plot of the morphology hybrid index consisting of leaf only characters separates the plants with GHIS of 0 from those with GHISs of 5 and 6 (Figure 2.12a). The MHISs of the hybrid plants range from 0 to 14. This completely encompasses the *B. oblongifolia* type plants, which do not share MHISs with a majority of the *B. robur*-type plants within the hybrid zone (the latter having MHISs of 15 and 16) (Figure 2.12b). The same pattern emerges when the morphological index includes inflorescence characters (Figure 2.13). Using this character array, there is complete separation of the parental type GHISs (Figure 2.13a), but the plants with hybrid GHISs occupy almost the full range of MHISs (Figure 2.13b.).

For ease of analysis, and because the use of the full range of scores on both hybrid indices violated the assumptions of the χ^2 model (i.e. sample sizes within each group were too small), each index was again subdivided and the data pooled. The genetic index was divided as was explained above: into *B. robur*-type plants (GHIS=0), hybrid or

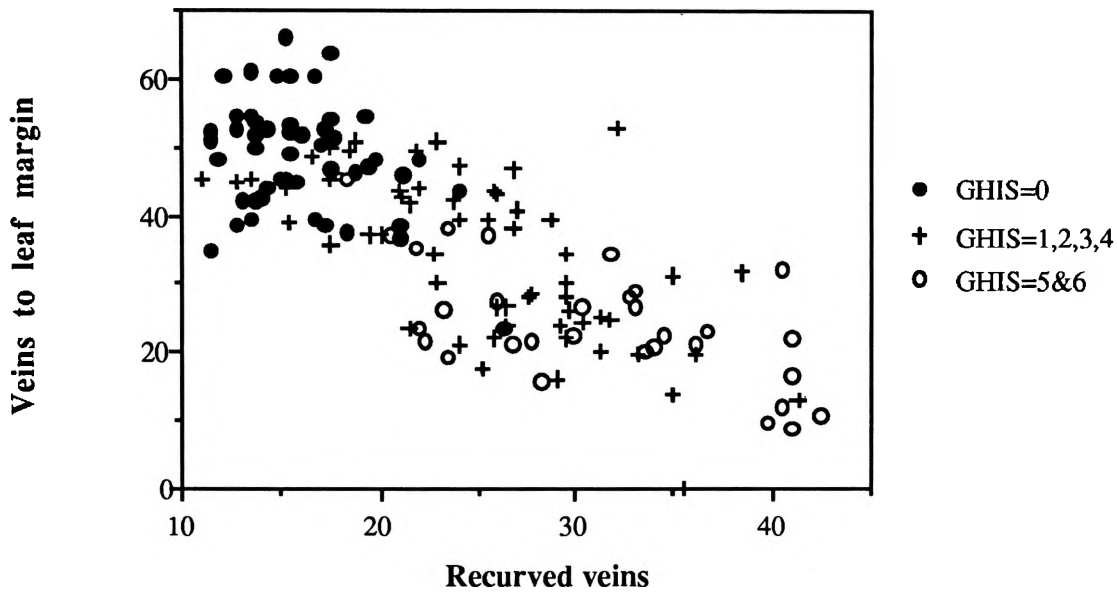


Figure 2.10. Plot of the number of marginal veins against the number of recurved veins for plants from the hybrid zones.

Figure 2.11. Stack column graphs of the size classes for each character measured from plants found in the hybrid zones. a. leaf length, b. leaf width, c. leaf area, d. petiole length, e. petiole width, f. serrations per centimetre of leaf margin, g. rachis length, h. rachis width, i. number of rows of flower pairs along length of rachis, j. number of flower pairs per whorl, and k. style length. For each graph, black bars are the plants with GHIS of 0, stippled bars are hybrid plants with GHISs of 1,2,3 and 4, white bars are plants with GHISs of 5 and 6.

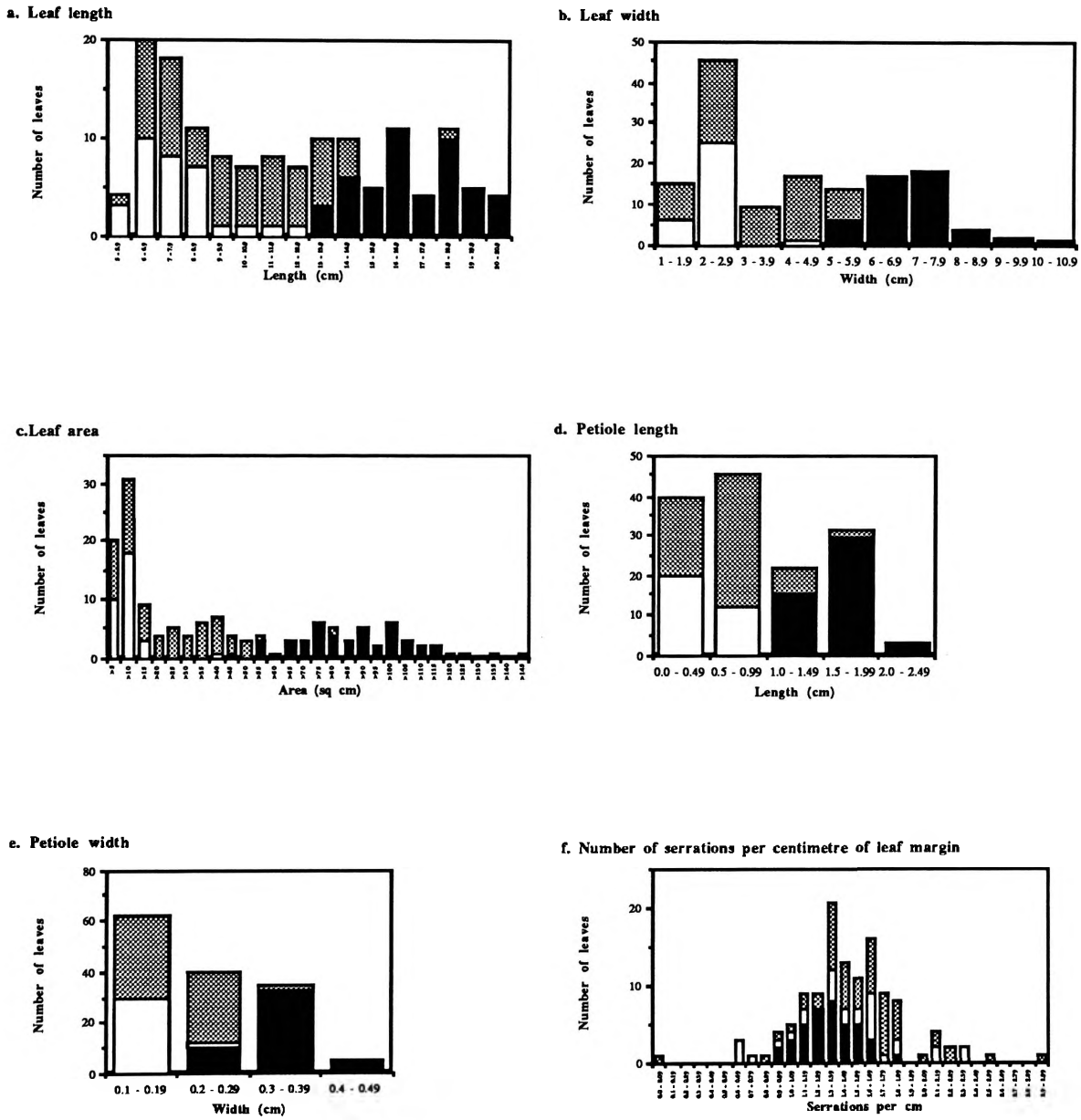
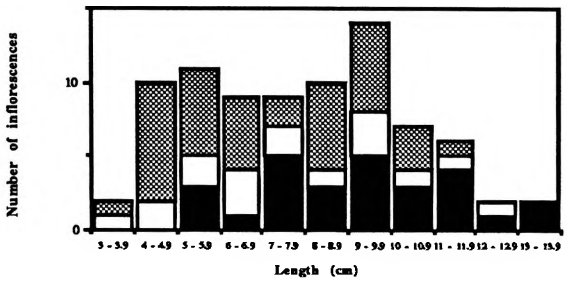
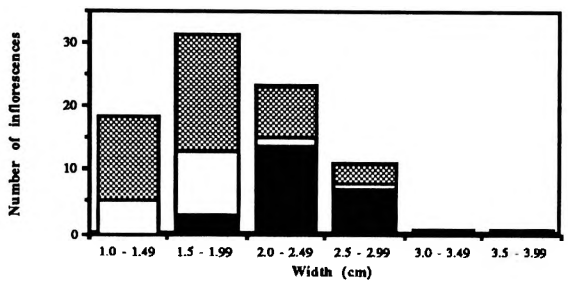


Figure 2.11 continued

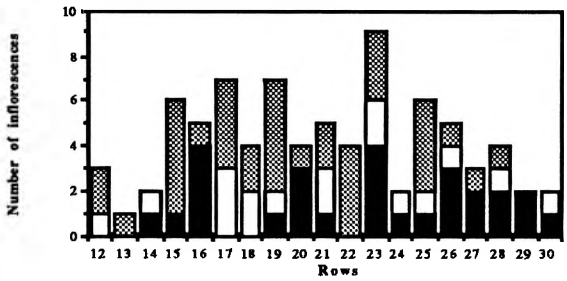
g. Rachis length



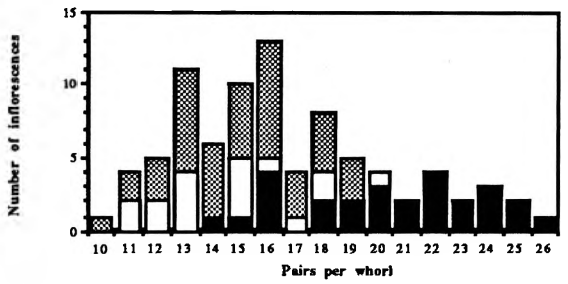
h. Rachis width



i. Rows of flower pairs along rachis length



j. Number of flower pairs per whorl



k. Style length

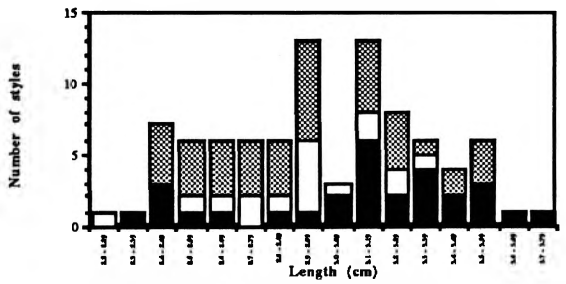
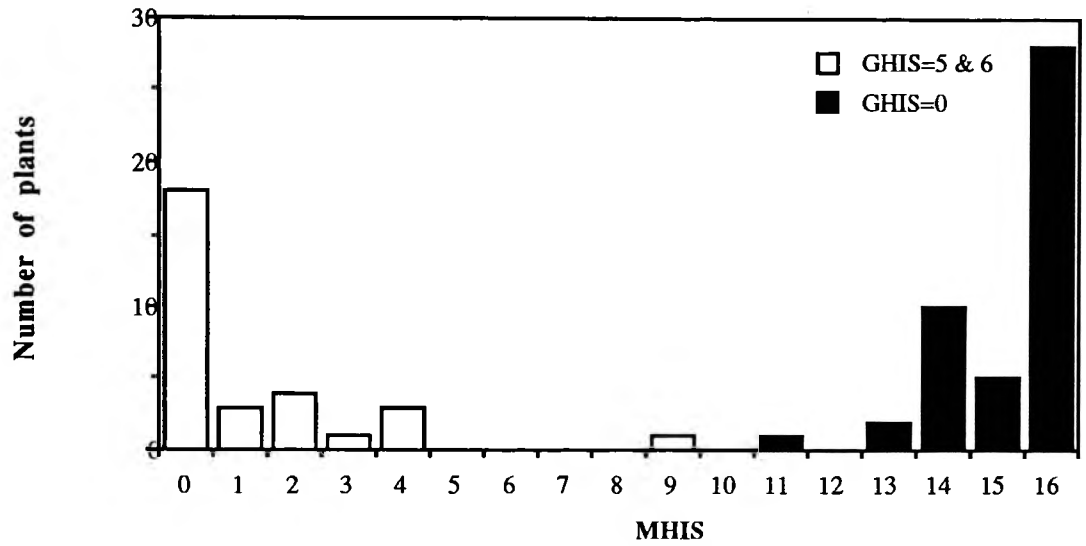


Figure 2.12. Stack column graphs of morphological hybrid index constructed from leaf characters only. a. histogram of plants from the hybrid zones with parental GHISs i.e. with genetic hybrid index scores of 0, 5 and 6. b. histogram of hybrid plants (GHISs of 1, 2, 3 and 4). Legends attached to graphs.

a. Plants with parental GHIS



b. Hybrid plants

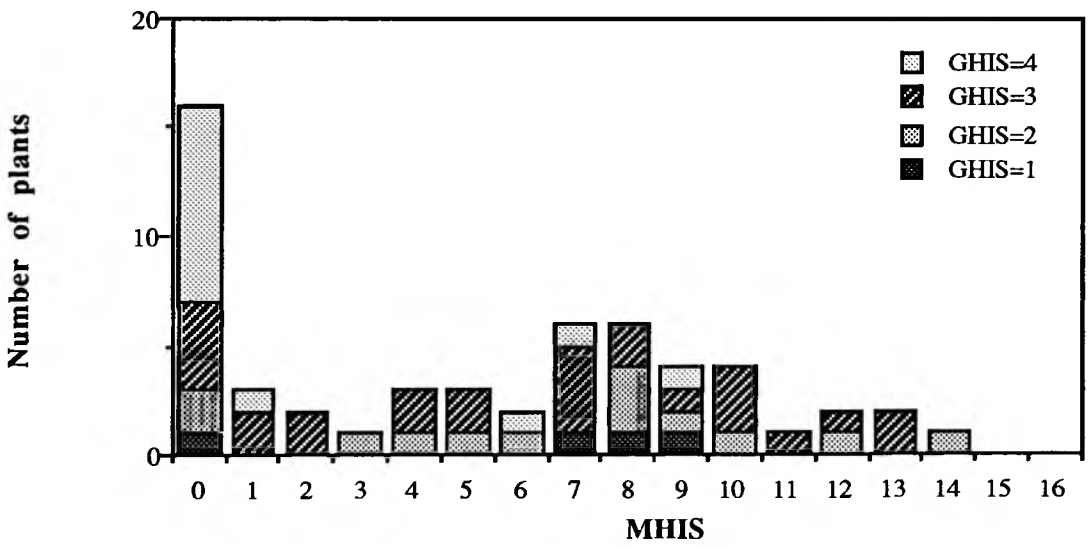
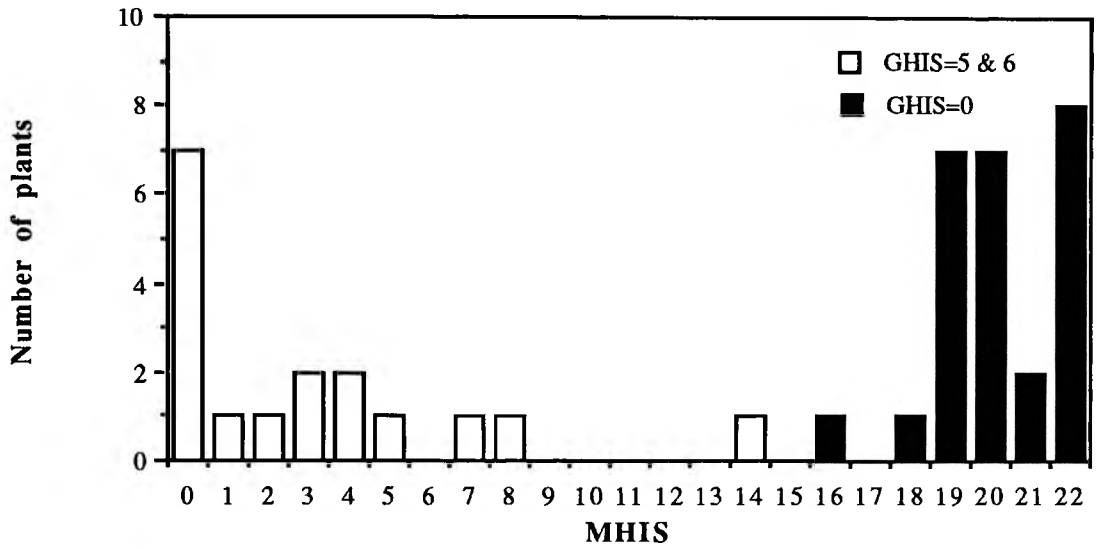
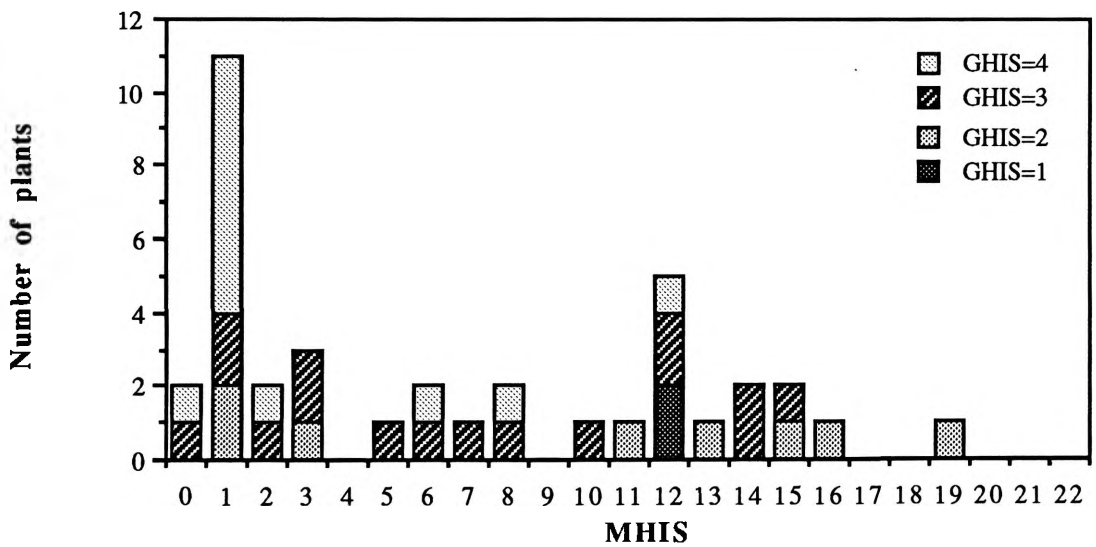


Figure 2.13. Stack column graphs of morphological hybrid index constructed from leaf and inflorescence characters. a. histogram of plants within the hybrid zones with parental GHISs i.e. plants with GHIS of 0 , 5 and 6. b. histogram of hybrid plants (GHISs of 1, 2, 3 and 4). Legends attached to graphs.

a. Plants with parental GHIS



b. Hybrid plants



intermediate plants (GHIS=1-4 inclusive) and *B. oblongifolia*-type individuals (GHIS=5 and 6). The morphological hybrid index was similarly divided: *B. robur*-type individuals (MHIS=12-16 inclusive), intermediates (MHIS=4-11 inclusive) and *B. oblongifolia*-type (MHIS=0-3 inclusive). A 3 x 3 contingency table was used to analyse these divisions, which detected highly significant heterogeneity amongst the cells (χ^2=145.5; p<<0.001). Subdivision of the contingency table revealed highly significant differences between all comparisons (R versus H χ^2=84.45, p<<0.001; R versus O χ^2=78.93, p<<0.001; O versus H χ^2=23.64, p<<0.001).

The perianth colour frequency histogram (Figure 2.14) shows that there is no overlap between *B. oblongifolia* types (GHIS=5 and 6) and *B. robur* types (GHIS=0) within the hybrid zone populations. Hybrid perianth colour varies over the whole colour range. Differences between colours associated with *B. robur*, hybrid and *B. oblongifolia* types were tested using a contingency chi-squared analysis. In order to do this, the colour spectrum illustrated in Figure 2.2 was divided into three colour intensities: light (0-5), medium (6-10) and deep (11-15). This showed a highly significant difference between the groupings in the colouring intensity (χ^2 = 73.893; p=0.0001). Contingency table subdivision revealed there were significant differences between the groupings (R versus H χ^2=45.818, p=0.0001; R versus O χ^2=48, p=0.0001, H versus O χ^2=8.965, p=0.0113).

2.3.3. Multivariate analyses

2.3.3.1. Discriminant Analysis

The discriminant functions generated indicated that the group of characters measured were adequate to separate the plants with parental GHIS, and are fairly accurate in correctly classifying plants with hybrid GHIS's. For brevity, the results of the final analysis (which included the whole character array) only are presented, although the other analyses were also performed.

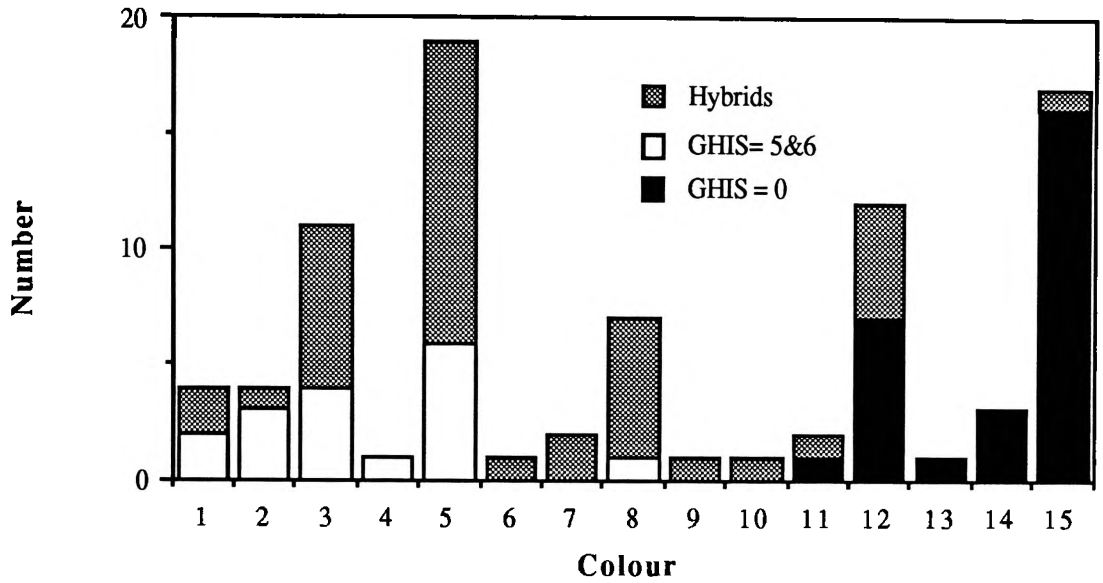


Figure 2.14. Stack column graph of perianth colour. The numbers on the abscissa correspond to those defined in Figure 2.3.

The discriminant function created using the total character array had excellent discriminatory power. The leaf characters contributed the greatest loading to the function, along with colour (which had the eighth highest loading at 0.808). Petiole length had the highest loading (with 0.94) and the number of serrations per centimetre of leaf margin had the lowest (with -0.291) (Table 2.5a). Further, the plants with GHISs of 0, 1, and 6 were all correctly classified. The plants with the remaining GHISs were correctly classified in greater than 50% of cases (Table 2.5b).

2.3.3.2. Principal Components Analysis

The Principal Components analysis performed on the data set comprising of only the leaf measurements extracted two factors. Component 1 accounted for the larger portion of the variation, explaining 85.1% of the total variance, while component two explained the remaining 14.9%. Within Factor 1, the leaf dimensions contributed most to the first component, with leaf length, width and length to widest point, as well as petiole width all having greater than 90% loading (Table 2.6a). All other characters showed more than 80% loading to the component, except for the number of serrations per centimetre of leaf margin, which had a negligible amount of loading. Number of serrations, however, contributed most of the variance on the second component, explaining greater than 90% of the variance, with minor contributions from both venation characters.

The first component explained most variance within the solution (Table 2.6b). This factor separated the plants collected from the pure stands and grouped them as either *B. robur* and *B. oblongifolia* (Figure 15a). This factor also satisfactorily grouped plants within the hybrid zones of hybrid indices of 0 form those with hybrid indices of 5 and 6 (Figure 2.15b). The intermediate plants were grouped between the two "parental-type" groups. Although there was some evidence that the more *B. oblongifolia*-like plants were grouped closer to the *B. oblongifolia* plant group, the plants with the hybrid GHISs could not satisfactorily separated from each other (Figure 2.15c). The second component did not group any of the genotypes.

Table 2.5. A summary of the discriminant analysis on the total character array of plants with GHISs of 0 to 6. Total sample size = 55. a. The loadings and the weights, indicating the relative discriminatory power of the character, are given. The integers in the first column under the loadings and weights are the ranking of each character. b. The classification matrix indicates the accuracy of the function at classifying plants into each GHIS. The actual GHIS is the actual GHIS of each plant determined using electrophoresis, while the predicted GHIS is that which each plant was grouped according to the discriminant function. N is the total sample size under each GHIS. Total classification accuracy was 74.4%.

a. Discriminant Function Solution

Character	Loading		Weight	
Leaf length	6	0.840	3	-2.129
Leaf width	3	0.919	12	-0.497
Length to widest point	7	0.822	13	-0.047
Petiole length	1	0.940	6	0.940
Petiole width	4	0.918	2	2.137
Serrations	14	0.291	5	1.200
Recurved veins	9	0.584	8	0.696
Marginal veins	10	0.541	4	-1.801
Leaf area	2	0.923	1	3.978
Rachis length	11	0.525	9	0.617
Rachis width	13	0.400	6	-0.822
Rows of flower pairs	5	0.848	7	0.806
Flower pairs per whorl	15	0.272	11	-0.503
Style length	12	0.459	14	0.013
Colour	8	0.808	10	0.578

b. Classification Matrix

		Predicted GHIS						
Actual GHIS	N	0	1	2	3	4	5	6
0	9	9	-	-	-	-	-	-
1	1	-	1	-	-	-	-	-
2	4	-	-	2	-	-	1	1
3	9	-	1	-	6	2	-	-
4	6	-	-	-	1	4	1	-
5	7	-	-	1	1	-	4	1
6	3	-	-	-	-	-	-	3

Table 2.6. a. Oblique solution structure for the Principal Components Analysis using leaf characters only. Underlined values show characters that appear to contribute most loadings to the factor. b. Variance contributions of the two factors extracted in the PCA solution in Table 2.9a. Direct variance refers to the proportion of the common variance the factor accounts for, independent of other factors, while joint variance is the variance that is shared with the other factor(s) (Hofmann & Simpson 1986).

a.

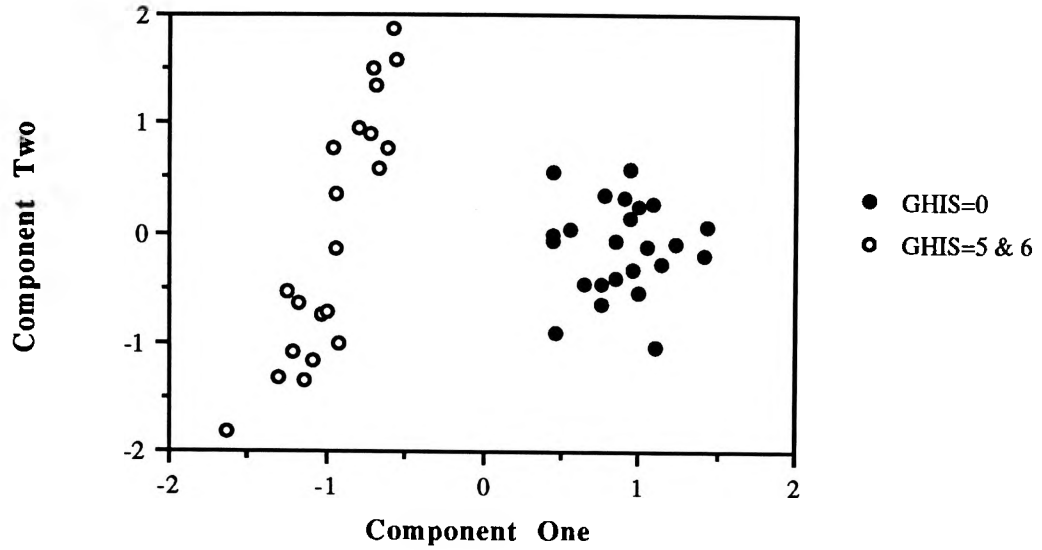
Character	Component One	Component Two
Leaf length	0.952	-0.081
Leaf width	0.953	-0.113
Length to widest point	0.944	-0.093
Petiole length	0.894	-0.155
Petiole width	0.933	-0.076
Serrations	-0.921×10^{-6}	0.957
Recurved veins	-0.829	-0.243
Marginal veins	0.896	0.310
Leaf area	0.859	-0.133

b.

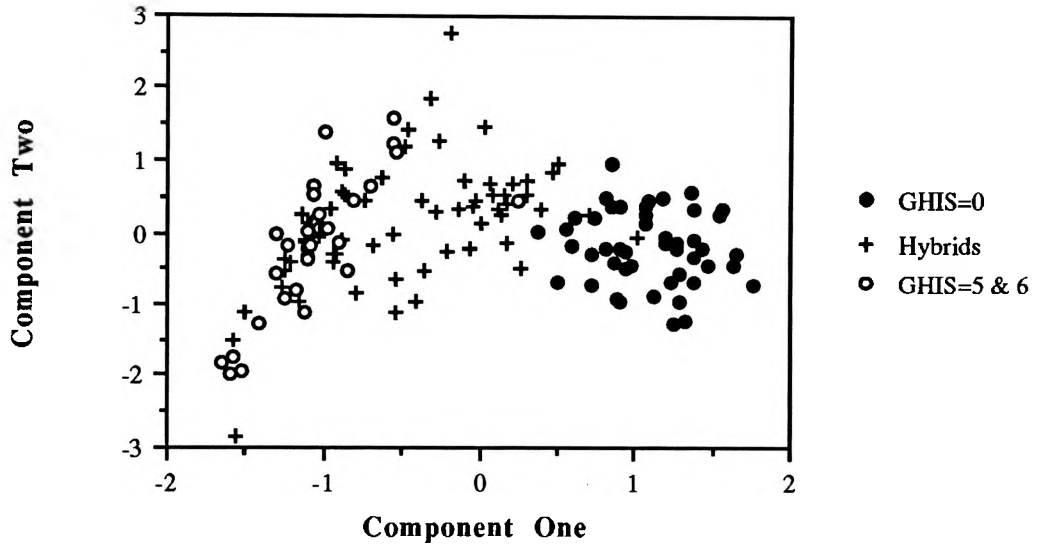
Variance Type	Component One	Component Two
Direct	0.857	0.149
Joint	-0.006	0.001
Total	0.851	0.149

Figure 2.15. (On following page) Plot of component one versus component two factor scores for the Principal Components Analysis performed using leaf characters only. a. The plot of pure stand plants only. b. Plot of all hybrid zone plants. c. Hybrid plants only. Legends are attached to graphs.

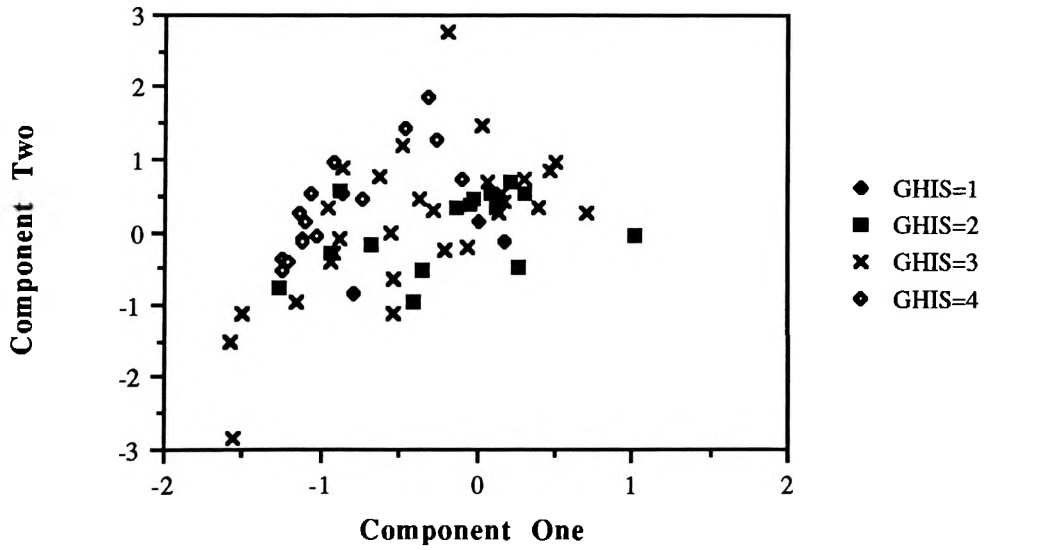
a. Pure stand plants



b. Hybrid zone plants



c. Hybrids



When the inflorescence characters were added to the leaf characters in the next analysis, three factors were extracted. The first component accounted for 60.6% of the variance (Table 2.7b), the leaf characters contributing mostly to this variance (Table 2.7a). The second component, accounting for only 28.3% of the variance, was characterized by inflorescence length characters (rachis length and the number of rows of flower pairs along the length of the rachis). The third component was influenced mainly by the number of serrations per centimetre of leaf margin, but only accounted for 11.1% of the total variance.

Only the scores for component one were plotted against those for the second component for this analysis, as the third component contributed little to the variance (Figure 2.16). The plot of plants from the pure stands only just separated the plants on the first component, but showed great overlap along component two (i.e. inflorescence length characters) (Figure 2.16a). The separation on component one was not apparent in the plot of the parental plants from the hybrid zones: separation along the negative diagonal of the plot shown (Figure 2.16b). The hybrids occur between the parental types. However, again these hybrids cannot be separated into smaller groups using these characters (Figure 2.16c).

The addition of colour to the data set did not alter the conclusions greatly, in that the leaf characters again contributed most to the first component (Table 2.8a), which accounts for 82.3% of the variance (Table 2.8b). Colour contributed substantially to the variance explained by component one, but substantially less than most of the leaf characters, six of which contributed about 90%. Again, the number of serrations per centimetre of leaf margin contributed most to the second component. Groupings were again better separated on the first component (Figure 2.17a), though intermediate genotypes could not be separated (Figure 2.17b).

Table 2.7. a. Oblique solution structure for the Principal Components Analysis using leaf and inflorescence characters. b. As for Table 2.6b.

a.

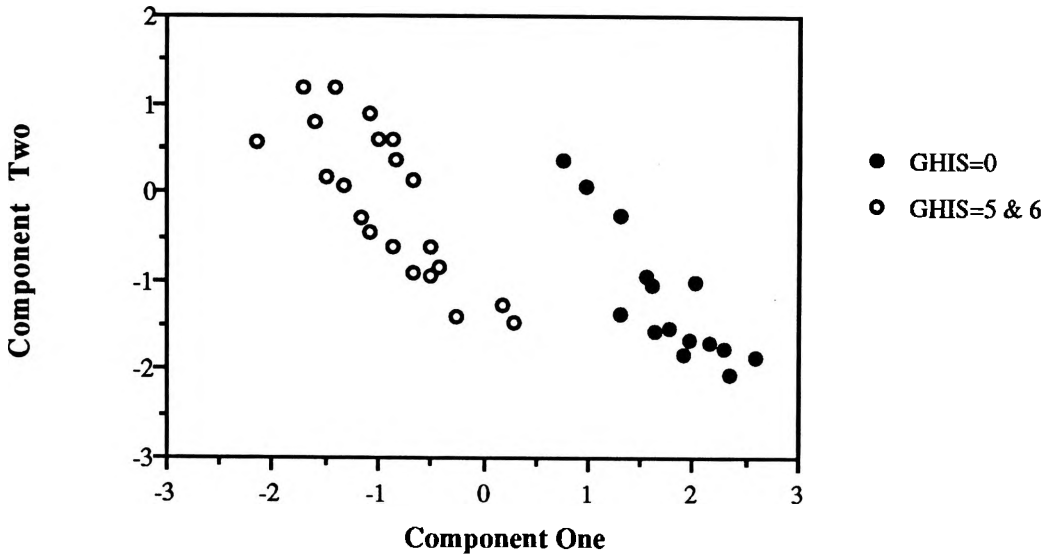
Character	Component One	Component Two	Component Three
Leaf length	0.701	0.074	-0.055
Leaf width	0.744	-0.002	-0.106
Length to widest point	0.704	0.063	-0.051
Petiole length	0.694	0.001	-0.147
Petiole width	0.740	-0.025	-0.091
Serrations	0.009	0.009	0.958
Recurved veins	-0.785	0.214	-0.234
Marginal veins	0.701	0.015	0.470
Leaf area	0.744	-0.002	-0.087
Rachis length	-0.253	0.848	-0.006
Rachis width	-0.021	0.393	-0.020
Rows of flower pairs	-0.368	0.890	0.137
Flower pairs per whorl	0.224	0.435	-0.020
Style length	0.141	0.394	-0.149

b.

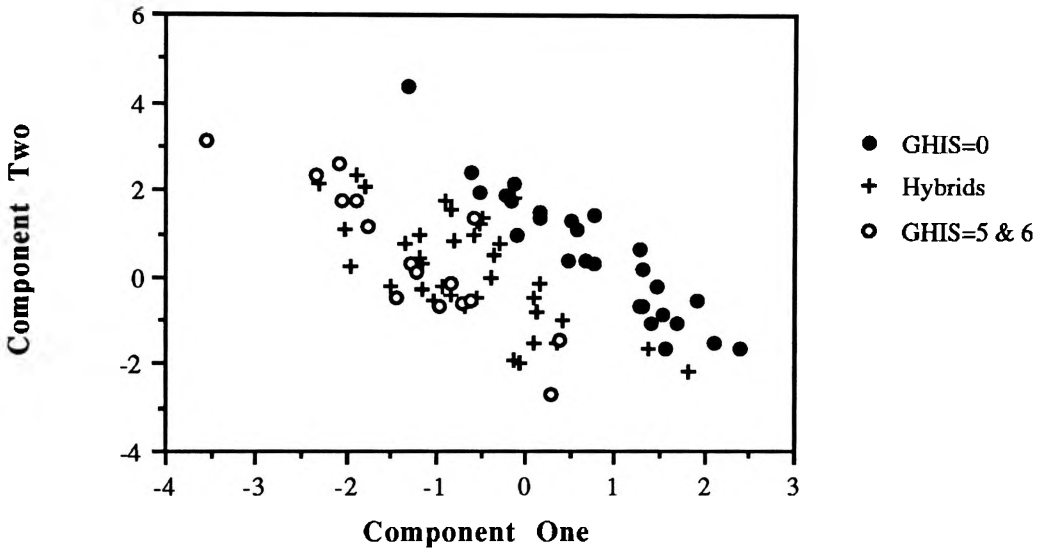
Variance Type	Component One	Component Two	Component Three
Direct	0.615	0.279	0.111
Joint	-0.0099	0.004	4.26×10^{-4}
Total	0.606	0.283	0.111

Figure 2.16. (On the following page) Plot of component one versus component two factor scores for PCA of leaf and inflorescence characters. a. The plot of pure stand plants only. b. Plot of all hybrid zone plants. c. Hybrid plants only. Legends are attached to graphs.

a. Pure stand plants



b. Hybrid zone plants



c. Hybrids

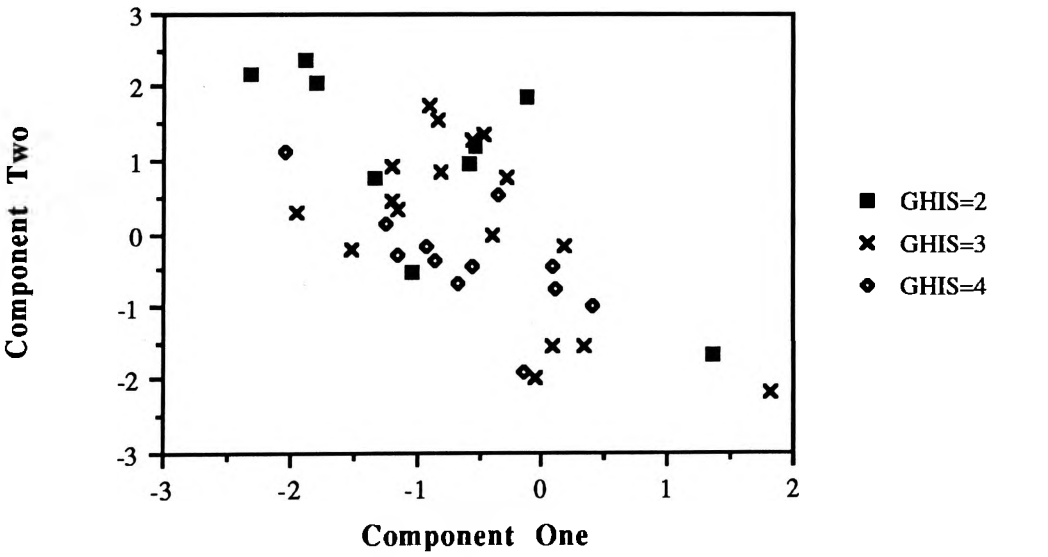


Table 2.8. a. Oblique solution structure for the Principal Components Analysis using leaf characters with the addition of colour. b. As for Table 2.6b.

a.

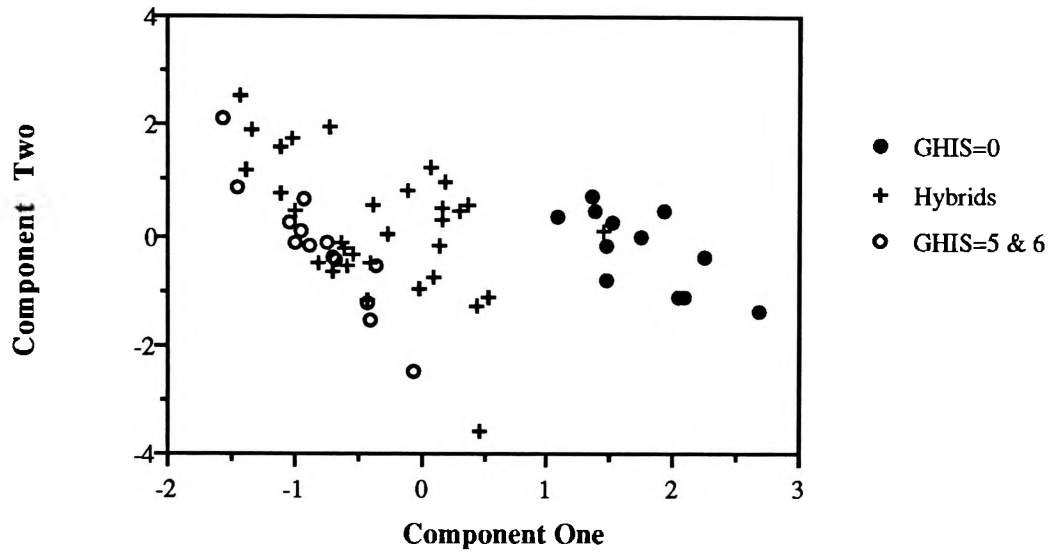
Character	Component One	Component Two
Leaf length	0.921	-0.052
Leaf width	0.932	-0.051
Length to widest point	0.899	-0.031
Petiole length	0.911	-0.124
Petiole width	0.898	-0.004
Serrations	-0.484	0.968
Recurved veins	-0.461	-0.368
Marginal veins	0.509	0.580
Leaf area	0.934	-0.047
Colour	0.765	-0.061

b.

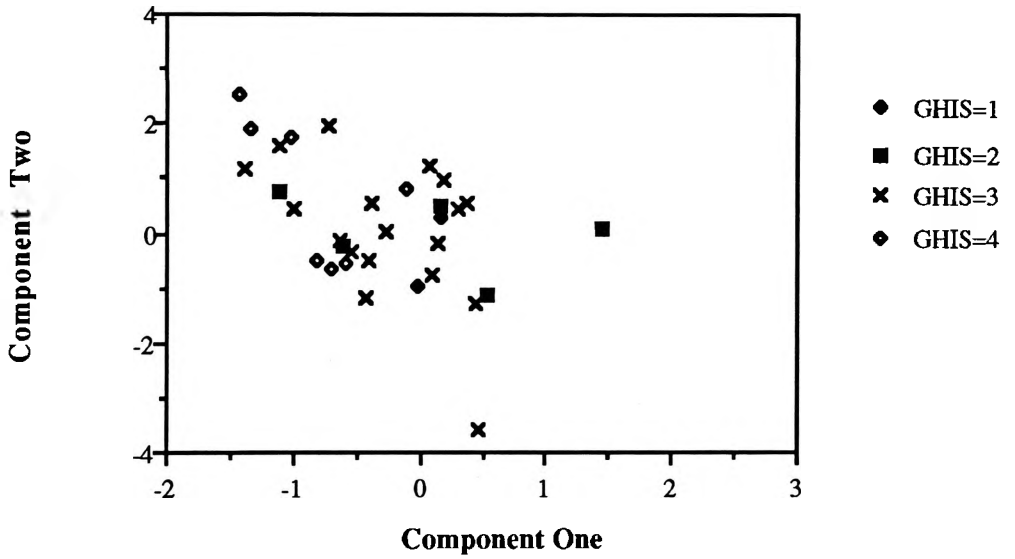
Variance Type	Component One	Component Two
Direct	0.789	0.179
Joint	0.034	-0.002
Total	0.823	0.177

Figure 2.17. Plot of component one versus component two factor scores for PCA of leaf characters and perianth colour score. a. Plot of all hybrid zone plants. b. Hybrid plants only. Legends are attached to graphs.

a. Hybrid zone plants



b. Hybrids



With the total character array used in the analysis, three factors were again extracted. Component One explained 56.4% of the variance (Table 2.9b), but leaf characters again contributed most to this variance (Table 2.9a). Component two accounted for 32.5% of the variance, and seemed to be explained by rachis length characters (the actual rachis length and the number of rows of flowers pairs along the length of the rachis).

Neither of these components satisfactorily separated the parental plants, but plotting the component one scores against the component two scores resulted in a parallel formation of points with the same gradient (Figure 2.18a). The plants with intermediate genotype again fell between the parental groupings, but again could not be separated in their own right (Figure 2.18b).

2.4. Discussion

This chapter suggests that there has been extensive hybridization of *B. robur* and *B. oblongifolia*, supporting the anecdotal evidence of hybridization between these two species (George 1981, 1987 and Taylor & Hopper 1988). Indeed, hybridization between *B. robur* and *B. oblongifolia* seems to be a common outcome in most regions in which they come in contact. Hybrids have been reported at 20 different sites throughout the range of the two species (Taylor & Hopper 1988): in several other sites within the Sydney Water Board catchment (pers. obs.), in the Royal National Park, south of Sydney (pers. obs.), in Ku-ring-gai Chase National Park (Elphinstone 1980), and in several sites around Brisbane (H.T. Clifford pers. comm. and pers obs.).

Further, there is evidence of backcrossing within the ecotone, resulting in a hybrid swarm, consisting of parentals, F_1 and F_2 hybrids and backcrossed individuals. There seemed to be a good correlation between morphological characteristics and genotype, i.e. morphologically intermediate plants were typically genetically intermediate. As predicted, leaf and colour characters were best at separating genetic groupings.

Table 2.9. a. Oblique solution structure for the Principal Components Analysis using total character array. b. As for Table 2.6b.

a.

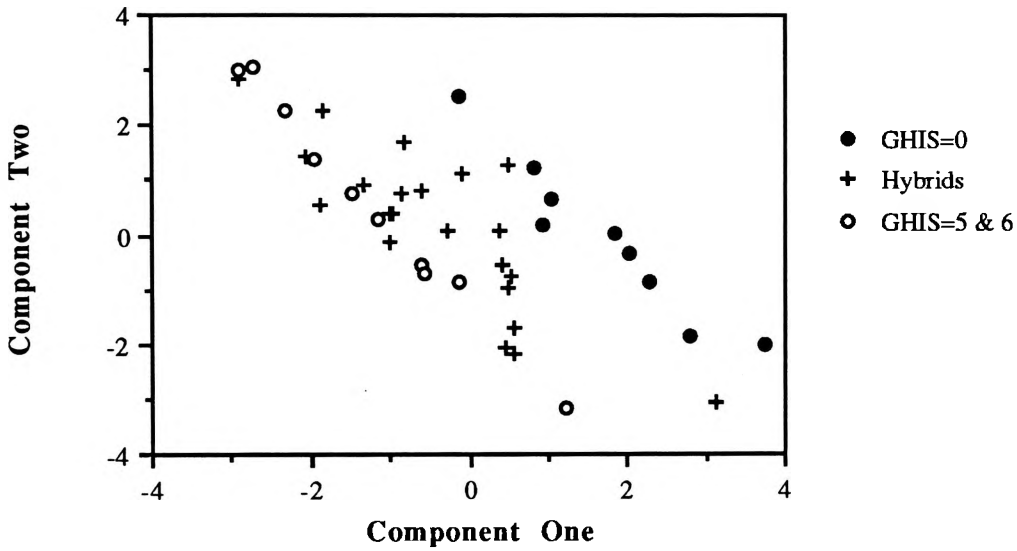
Character	Component One	Component Two	Component Three
Leaf length	0.511	0.126	-0.004
Leaf width	0.614	-0.008	-0.083
Length to widest point	0.567	0.053	-0.010
Petiole length	0.593	-0.031	-0.092
Petiole width	0.630	-0.041	-0.051
Serrations	-0.045	0.040	0.934
Recurved veins	-0.679	0.338	-0.129
Marginal veins	0.460	0.097	0.679
Leaf area	0.611	0.001	-0.067
Rachis length	-0.210	0.763	0.065
Rachis width	0.029	0.266	-0.323
Rows of flower pairs	-0.400	0.849	0.033
Flower pairs per whorl	0.273	0.273	-0.124
Style length	0.001	0.495	0.026
Colour	0.492	0.020	-0.028

b.

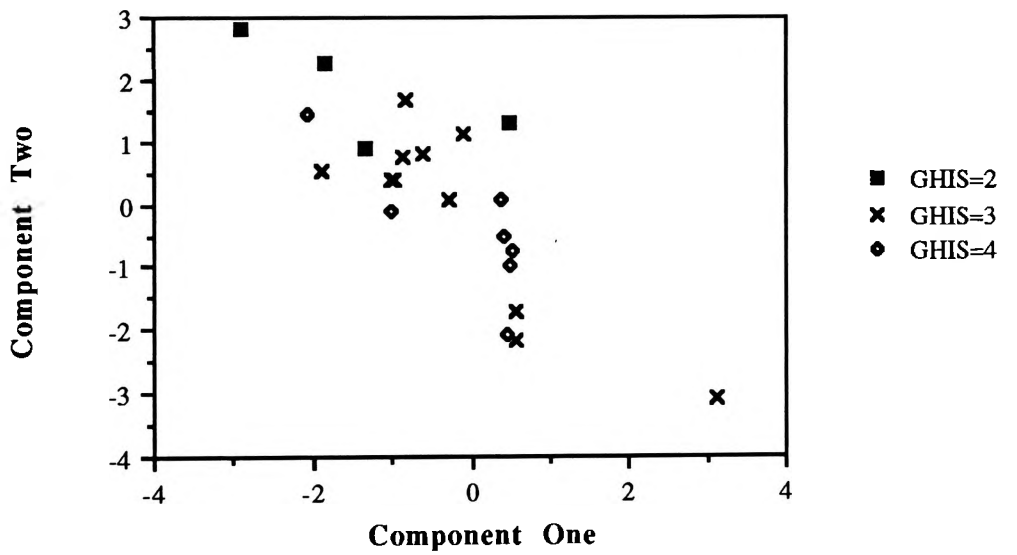
Variance Type	Component One	Component Two	Component Three
Direct	0.575	0.320	0.111
Joint	-0.011	0.005	-1.17×10^{-4}
Total	0.564	0.325	0.111

Figure 2.18. Plot of component one versus component two factor scores for PCA of total character array (leaf and inflorescence characters and perianth colour score). a. Plot of all hybrid zone plants. b. Hybrid plants only. Legends are attached to graphs.

a. Hybrid zone plants



b. Hybrids



2.4.1. Genetic Variation

There was little intraspecific genetic variation detected within the allopatric populations of the two species of *Banksia* surveyed: only four loci in the 32 enzymes surveyed showed any variation, and this variation was limited to *B. oblongifolia.* This low level of variation seems to be common in many *Banksia* species. Scott (1980) detected variation in three loci for *B. menziesii* and two loci for *B. attenuata* out of 13 enzymes tested. Carthew *et al.* (1988) reported that four loci out of the 24 enzymes tested for *Banksia spinulosa* revealed variation, while only one variable locus was detected from the same array of enzymes for *B. paludosa.*

Although there was little intraspecific variation (and no variation detected within *B. robur*), three of the four variable loci detected in this study, *Adh* , *Sod* and *Gdh*, showed virtually fixed differences between the species, and thus proved useful in separating the two species. Using these genetic differences, pure stand *B. robur* could be separated unequivocally from pure stand *B. oblongifolia.* These genetic differences could not, however, differentiate the parental individuals from plants with a hybrid origin, within the hybrid zones. The extensive introgression suggested by the allele frequencies of plants within the mixed stand could mean that some of the plants within the hybrid zone with "parental" GHISs may have been produced through backcrossing. Further, the genetic variation within the pure stands of the *B. oblongifolia* complicated the definition of *B. oblongifolia* within the mixed species stands. A plant within the hybrid zone with a GHIS of 5 may either be a "pure" *B. oblongifolia* or be of hybrid origin. It also follows that a plant with a GHIS of 2 may be an F_1 generation plant or may be part of the F_2 generation or beyond. The difficulty of *B. oblongifolia* recognition is illustrated by the reduction in the proportion of plants with a GHIS of 6 in the hybrid zone. In the pure stand *B. oblongifolia*, the plants with GHIS of 5 roughly equalled those with GHIS of 6, a ratio that would also be expected within the hybrid zone, if the sample of plants was randomly selected. The lack of pure *B. oblongifolia* may indicate the selection against these plants

within the wetter ecotone environment. An alternative explanation is the increase in the number of plants scoring 5, as a result of the increased opportunity for backcrossing.

The plants surveyed electrophoretically in the hybrid zones had a range of genotypes: the genotypes found in the pure stand parental populations as well as a range of intermediate genotypes. There was a dominance of plants with GHIS of 0, which may suggest that *B. robur*-type plants are more fit within this region. There was a relatively small proportion of plants with GHIS of 6 (discussed above), with a larger proportion of plants scoring 5. A fairly high proportion of plants with GHIS of 3 indicates that there has been an exchange of genes between the parental types in the hybrid zone. Further, the occurrence of smaller proportions of plants with GHISs of 1, 2 and 4 suggests that the hybrids are reproductive and that there is interbreeding between the hybrids and hybrids and parental types, although determining the extent to which this occurs is difficult from only three loci.

2.4.2. Morphological Characteristics

It has been suggested that caution should be used in labelling as "hybrids" individuals with intermediate morphological features, found in suspected hybridizing populations. There is a real possibility of phenotypic plasticity "creates" intermediate morphologies, and this can potentially confound the interpretation of the hybrid zone structure (Bradshaw 1965). Further, an individual with an intermediate genotype may not necessarily always be morphologically intermediate. This problem is clearly demonstrated in the study by Woodruff and Gould (1987) on the land snail, *Cerion*, in which individuals identified morphologically as hybrids were restricted to a small area, while individuals, identifiable as hybrids only through allozymes, were actually more widespread. Studies (e.g. on *Pseudophryne* [a southeastern Australian frog] [McDonnell *et al.* 1978] and on *Thomomys* [pocket gophers] [Patton *et al.* 1979]) have found that allelic frequencies and morphology do not correlate.

In this study, the genotypic differences between plants within the pure stands were associated closely to differences in morphology. This was particularly apparent for leaf characters, which correctly predicted the GHIS of plants within both pure stands, and the parental plants within the hybrid zone. The plants with intermediate genotypes within the hybrid zone had intermediate morphological characters. However, the morphological characters could not consistently predict the plants with intermediate GHISs, suggesting that the patterns of inheritance within the complex are more complicated than simple hybridization of parental genotypes, resulting in correlated morphological, genetic and environmental clinal variation from one parental extreme to the other. Another explanation may be that some of the morphological characters may be more plastic than may have been originally suspected. This is suggested by the comparison of the morphological characteristics of the plants in the pure stand and hybrid zone populations, the latter of which show greater morphological variation within parental GHISs. The genetic complexity of the plants within the hybrid zones is perhaps evidence that the hybrid zone is older than one or two generations old.

If there is a high level of hybrid fertility within the two hybrid zones studied, with no restriction to reproduction between parents, between hybrids and between hybrids and parental types, and no apparent breakdown in reproductive ability within any of the products, this complex can be considered to be one "syngameon" (Grant 1981). This is similar to the conclusion reached by Elphinstone (1980). However, because the species are ecologically and morphologically distinct, taxonomically the present specific epithets are more practical.

Conventional species theory suggests that the products of interspecific fertilization are not self perpetuating (they are either selected against or are unreproductive) (Darwin 1859), and this lack of fecundity may reinforce reproductive barriers between the hybridizing species (by acting as a gene sink) (Hewitt 1988). There are, in fact, many examples of hybrid zones with infertile hybrids. However, there are also an increasing number of

studies reporting at least limited hybrid viability and fertility, resulting in extensive backcrossing within hybrid zones in many systems (e.g. *Thomomys* [pocket gophers]: Thaeler 1968; *Quiscalus* [the common grackle]: Yang & Selander 1968; asymmetrically in *Gryllus* [a North American field cricket]: Harrison 1986; *Iris* [Louisiana iris]: Arnold *et al.* 1990). This study presents data suggesting that there is backcrossing within *Banksia*, resulting from hybrid fertility. If direct evidence of hybrid fertility can be gathered (seed germination), *Banksia* could also be added to the list.

2.4.3. Species and hybrid recognition

The discriminant analyses show that the leaf characters were better at grouping plants with the same GHISs than characters measured from the inflorescence. However, the discriminatory power of the function generated from the analyses was greater with the larger number of characters included. These results agree with Hair *et al.* (1987), who suggested that differences in the combination of characters used to calculate the function result in variation in the loadings of a character from function to function.

B. robur-type and *B. oblongifolia*-type plants could be separated satisfactorily using the morphological characters measured. However, the genetically intermediate plants were not so easy to separate using these characters. The greatest success was achieved using the full character array, although this resulted in 38% of hybrid plants being misclassified. This suggested that there is a range of plants with a great variety of morphological traits that are not tightly controlled by the genetics, and further, that this group of plants may represent the produce of many generations.

Principal Components Analysis has often been favoured by analysts of morphological differences between the hybridizing species, because the variables used in the analysis are weighted consistently, even after the removal or addition of variables in subsequent analyses (Moore & Buchanan 1985). Other studies have suggested that caution is

necessary, but that the use of PCAs in determining characters that contribute most to defining groups within a population is appropriate (Adams 1982).

In agreement with the discriminant analyses, regardless of the combination of characters used in the matrix, all PCA analyses suggested that leaf characters, particularly leaf size and venation patterns, separated the parental plants, according to the genetic markers, in both the pure populations and the hybrid zones. The individuals with intermediate genotypes, were also intermediate in morphology. This was the extent of the reliability of the morphological characters, as further and satisfactory separation of the intermediate genotypes was not possible.

2.4.4. Introgression

The range of genotypes intermediate to the parental species and the probable F_1 hybrids (revealed by allozyme electrophoresis) in the hybrid zones indicate that there hybrids are fertile and freely backcrossing. However, judging from the comparatively small proportion of plants with these introgressed genotypes, the level of this backcrossing may, so far, have been fairly limited. Introgression is also suggested by the continuum of morphological characters measured from the plants within the hybrid zones. Discriminant analyses, based on morphometric characters, separated the parental-type plants, but, in many instances, could not group the genetically intermediate plants into their correct GHIS.

The "*B. robur* alleles" detected within the allopatric populations of *B. oblongifolia* may provide further evidence of introgression within this complex. The presence of these alleles within pure *B. oblongifolia* stands may be the result of one or more immediate and long term explanations. Firstly, dispersal from one species to the other may be more frequent and of greater distance than was expected, as there is about one to two kilometres between the pure populations surveyed. This genetic pattern also suggests that there is an asymmetry in the dispersal pattern of the pollen or seed, or a very strong

barrier to the flow of *B. oblongifolia* genes into the *B. robur* populations. This suggestion of asymmetry will be further explored in the next Chapter.

Alternatively, the *B. robur* alleles present in the *B. oblongifolia* pure populations may in fact be a relict from past hybridization events. Anderson (1953) suggested that the most common outcome of the formation of hybrid swarms is the incorporation of the genes of one of the hybridizing species into the other. Alleles from one species have been found in individuals of another species, well distant from the hybrid zone (Sage & Selander 1979; Harrison 1986). Such occurrences have been attributed to the mixed ancestry of the species, the gene pool of which diverged to give rise to the species presently hybridizing.

Determination of the selection and the level of gene flow within the hybrid zone may add support to the suggestions discussed above as to the origin of the hybrid zone and the apparent introgression between the two species. Whereas gene flow can influence the potential makeup of the population, the selection acting on the plants can shape the hybrid zone, controlling the type of plants that survive to maturity. Detection of selection is more difficult than detecting gene flow, as only the survivors of the process can be directly observed - it is difficult to assess the characteristics of the type of plants that were selected against. The following Chapters will attempt to address the questions of the level of selection and the extent of gene flow within the hybrid zone formed by these two species.

Chapter Three

Selection within the *B. robur/B. oblongifolia* hybrid zone.

3.1 Introduction

The presence of genetically intermediate plants in natural stands of *Banksia robur* and *B. oblongifolia* was described in Chapter Two. Further, the genetic composition of these stands is complex, and includes both F1 hybrids and later generation plants that are genetically more similar to one or other of the parental species. However, to infer the stability or the longevity of the hybrid zone, it is important to determine the spatial relationship of these genetically diverse individuals. The spatial arrangement and the interaction between the individual plants, determine the evolutionary forces acting on and shaping the distribution of the species. These forces come in the form of selection acting on the different phenotypes, resulting in genotypic clines.

Certain characteristics describing the spatial pattern of the cline (usually referred to as the "cline shape" [Szymura & Barton 1986]) are dictated by the combination of dispersal of the genes within the system and the selection acting upon the hybrid genotypes and phenotypes. In turn, therefore, the shape of the cline has the potential to reveal much about the dynamics of the hybrid zone, if used in conjunction with other traits of the species under study (Kocher & Sage 1986).

3.1.1. Consequences of the selection regime on hybrid zone formation.

Within the many definitions of hybrid zones outlined in Chapter One, there are many theories that attempt to explain the stability of these zones. The common thread connecting these theories is the level of fitness exhibited by the plants of hybrid origin, which can roughly be divided into two different areas: (i) hybrids have a higher level of

fitness than the parental plants in certain areas (such as ecotones) (espoused by Endler 1977; Moore 1977; Levin & Schmidt 1985), and (ii) hybrids are usually selected against, but the hybrid zone is maintained through continual recruitment of hybrids (Barton & Hewitt 1981). On this basis, it may be expected that the hybrids formed in the hybrid zone may show either a reduction or increase in fitness and potential reproduction. In the first theory, the hybrid zone would be restricted in size by environmental constraints, while in the second, by the balance between dispersal and selection.

3.1.2. Determination of selection within a system

The level of selection acting on certain genotypes or phenotypes within a system can be determined through several means (Endler 1986). Three will be attempted in this Chapter to determine the level of selection acting on the hybrids in this system: (i) detection of the deviation of genotype frequency from those expected under formal hypotheses; (ii) use of parameters derived from the change in gene frequency along a hybrid zone; (iii) the direct measurement of phenotypic characters upon which selection may act.

3.1.2.1. Deviation from formal null hypotheses

One of the most common formal hypotheses used in detecting selection on a single locus is determining deviations from gene frequencies expected from random mating, or Hardy-Weinberg equilibrium. Deficits of a genotypic class compared to the proportions that are expected under Hardy-Weinberg equilibrium may indicate the strength of selection against individuals belonging to that class, providing that the normal assumptions of random mating, Mendelian inheritance and minimal migration and mutation are met. The selection against hybrids in a system may be indicated by the deficit of hybrid or heterozygote genotypes, while selection favouring these genotypes within an area would result in heterozygote excesses.

Another of the formal null hypotheses to be used in detecting selection within a system is to determine the level of associations between loci. Strong linkage disequilibrium is often

detected in association with a cline (Kocher & Sage 1986, Szymura & Barton 1986, Howard & Waring 1991, Szymura & Barton 1991). The maintenance of this strong disequilibrium is thought to be through the constant movement of parental genotypes into the centre of the cline (Slatkin 1975), or through greater fitness of parental combinations of alleles.

3.1.2.2. Parameters derived from cline shape

Another way to detect selection on a system is to examine the shape of the cline formed by a variable trait and to derive a number of parameters. These parameters can then be used to calculate selection coefficients for the population. The two simplest parameters that are useful in describing a hybrid zone are the position of the cline and its width. The former simply places the cline in space, and is usually designated and determined by the geographic position along the cline where the frequency of the trait being measured is 0.5. This measure is useful for the comparison between clines of different traits within the one hybrid zone.

Cline width can be used to determine the potential dispersal and selection within the system. Within the *B. robur*/*B. oblongifolia* system, estimates of the distance between the *B. oblongifolia* woodland and the *B. robur* marshes (pers. ob.) and other work (Keith and Myerscough 1993) suggest that the width of the cline would be no more than 100 metres.

Beyond the region of the hybrid zone designated as the cline, there are often regions where the allele frequency tends towards 0 or 1. These are known as the tails of the cline, and indicate the rate and degree of introgression within the system. The rate of decay of the tail towards 0 or 1 indicates the strength of selection on an allele. If the decay of the tail is gradual (close to 0), the selection against the allele will be weak, enabling the allele to penetrate the genome of the other species. Similarly, the length of the tail also indicates the strength of selection, or the barrier to the allele. A long tail (much greater than the

width of the cline, for example) indicates a low level of selection, again allowing the passage of the allele into the alternative species' genome. For the hybrid zone formed by *B. robur* and *B. oblongifolia*, the genetic survey of the species presented in Chapter Two indicated the presence of what was designated the "*B. robur* allele" within the pure *B. oblongifolia* populations, but not *vice versa*. This may suggest the ease of passage of the *B. robur* allele into *B. oblongifolia*, which should be indicated by a long, slowly decaying tail tending toward 0. The nature of the *B. robur* tail is difficult to predict, but it is expected not to be as long as the *B. oblongifolia* tail, because there was no evidence of *B. oblongifolia* alleles in the pure populations of *B. robur* surveyed in Chapter Two. The differences suggest an asymmetrical cline.

These cline shape parameters can be used to determine the rate of dispersal rate of genes within the pool and the level of selection against these genes. The formulae for arriving at these estimate are presented in Section 3.2.3.

Selection need not be constant across loci (e.g. Heywood 1986). Indeed, Barton (1983) emphasized the need for multi-locus studies of variation across a hybrid zone. Variation in regimes of selection will be manifested as differences in the width of the cline (Barton 1983, Szymura & Barton 1986). These inter-locus differences also suggest the importance of determining the recombination patterns of the loci within the population (Weir 1979). Even if there are no detectable differences between loci, the importance of looking at the loci individually remains, because the cumulative effect of the characteristics of the loci may be different to the sum of the individual effects (Barton 1983).

3.1.2.3. Phenotypic variation within a hybrid zone

A method of detecting the level of selection occurring within a system, which is independent of genetic variation, is direct measurement of characters describing survivorship, fitness and fecundity within a population (Endler 1986). This was

attempted in this study on representative characters, which were used to determine differences in vigour and fecundity between genotypic classes. Differences in these characters were also determined in order to detect spatial variation in vigour and fecundity between groups of *B. robur*, *B.oblongifolia* and hybrid origin plants. It is expected that if there is hybrids exhibit selective advantage within the ecotone, they will at least be more vigorous and numerous, and may exhibit a higher degree of fertility.

This Chapter presents an analysis of parameters describing cline shape for the change in allele frequency across the hybrid zones in both the Cataract and Darkes Forest sites. From these parameters, estimates for dispersal rate and selection pressure on the sampled loci within the hybrid zone populations. Plant traits, describing vigour and fecundity, were recorded for the groups of plants described as *B. robur* type, hybrid origin or *B. oblongifolia* type, and differences in these traits are observed through the hybrid zone, to determine if vigour and fecundity of the groups are related to the position of the plant within the hybrid zone.

3.2 Methods

3.2.1. Sites and sampling

The study described in this Chapter was conducted in the two hybrid zones in Darkes Forest and the Cataract catchment (see Chapter One). The position of the tagged plants within each site was mapped in order to determine the spatial distribution of parental and hybrid genotypes within each hybrid zone site (Figures 3.1 and 3.2).

Transects were established in each population to simplify the observation of the change in genotype and phenotype through the hybrid zone. A series of adjacent quadrats was surveyed along these transects, each quadrat measuring 10m wide x 5m long. The frequency of the alleles which were fixed in *Banksia robur* (i.e. Adh^f, Sod^s and Gdh^f) were calculated for each quadrat, using the genotypes of the individual plants determined in Chapter Two.

Figure 3.1. (On following page) Distribution of plants with each GHIS within the Cataract site. The closed circles show the position of plants that were genotyped within the population, while the open circles were plants that could only be classified using morphology. Legend is attached to the Figure. The site is bounded in the east by a fire trail, in the south-east and south by the creek running through the swamp, and on the north and west by woodland.

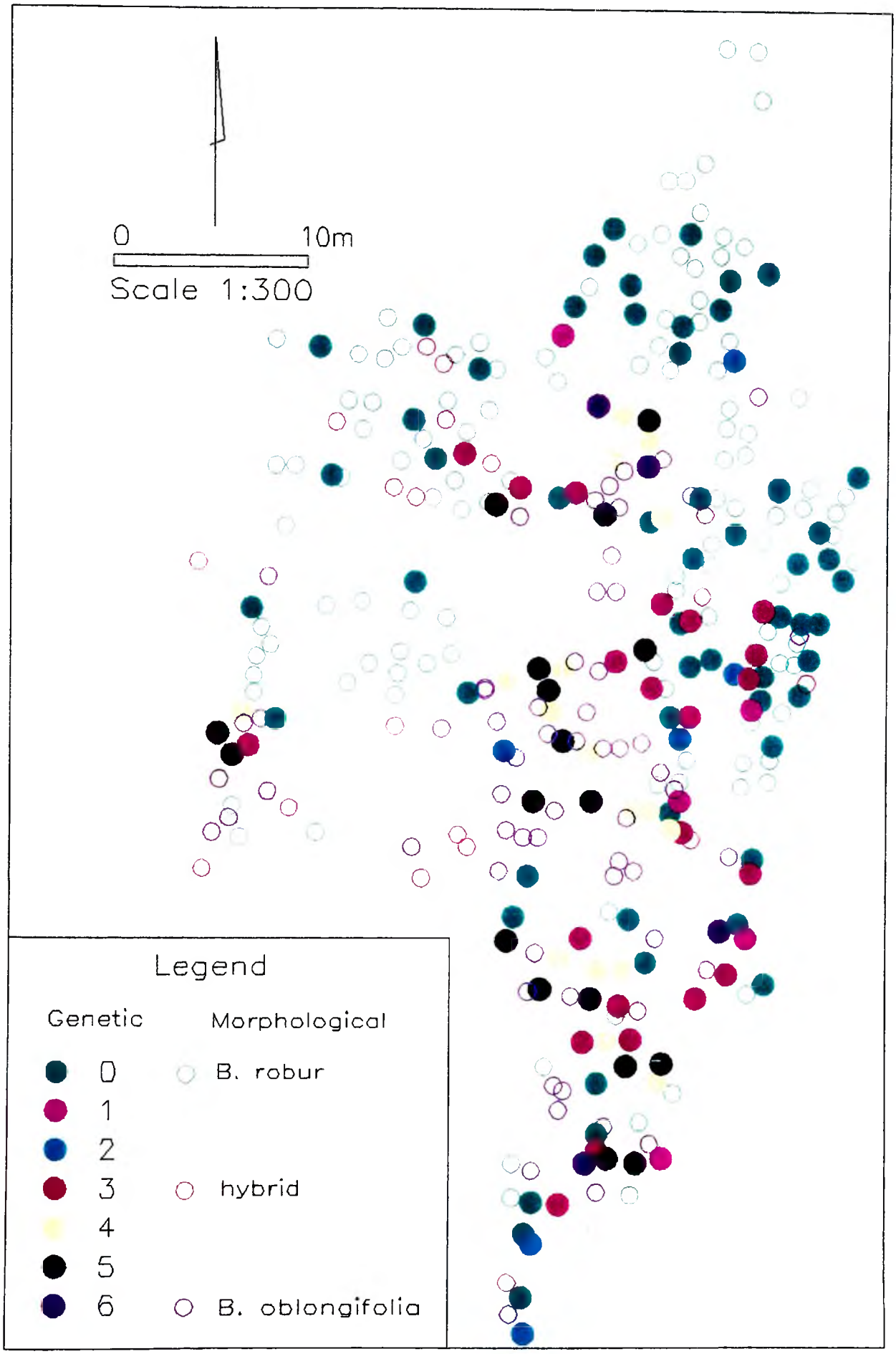
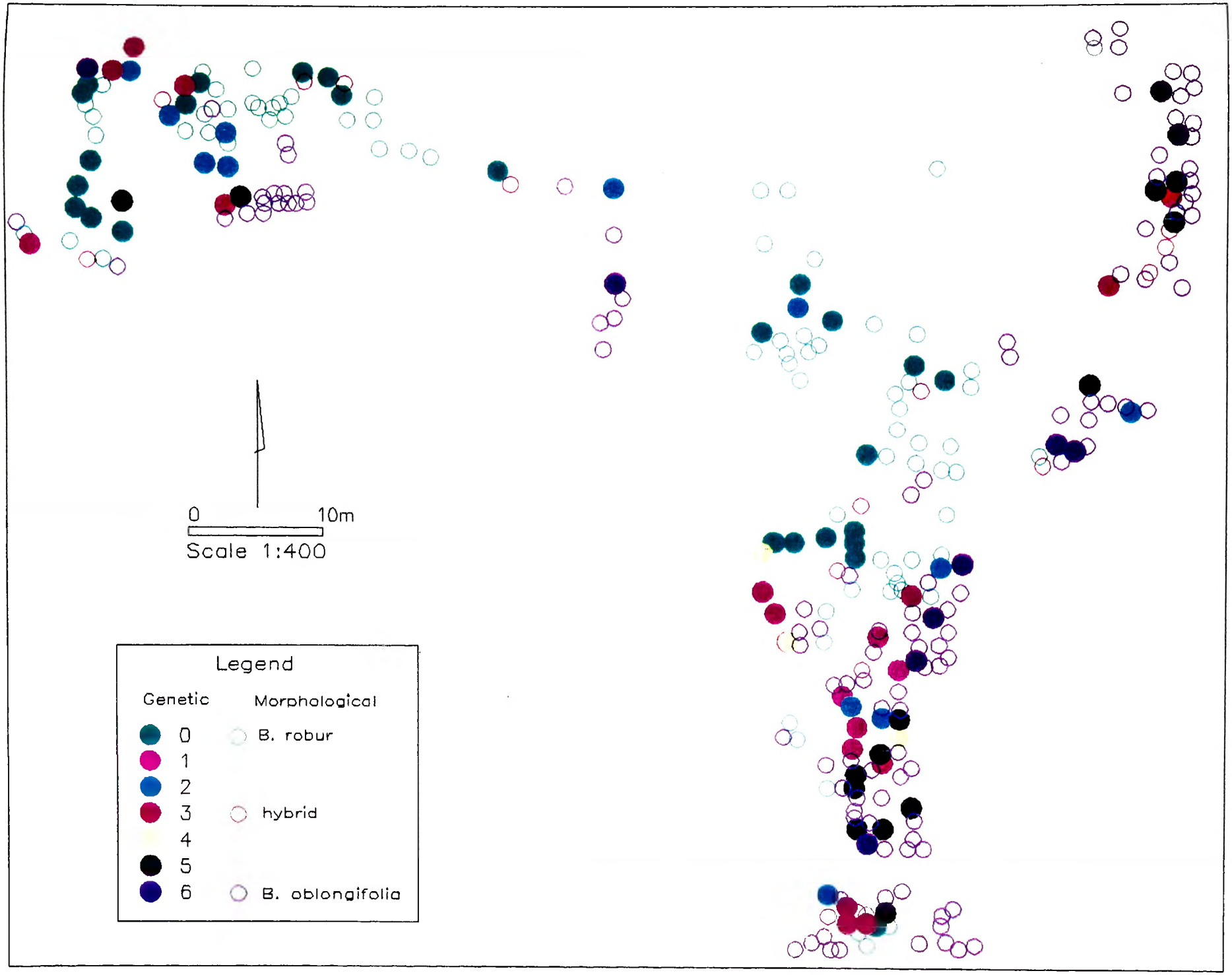

0
10m
Scale 1:300
Legend
Genetic
Morphological
0
1
2
3
4
5
6
B. robur
hybrid
B. oblongifolia

Figure 3.2. (On following page) Distribution of plants with each GHIS within the Darkes Forest site. As for Figure 3.1, legend is attached to the Figure. The site is bounded in the north by a fire trail cut for seismic surveys, and in the south, east and west by woodland.


0
10m
Scale 1:400
Legend
Genetic
Morphological
0
1
2
3
4
5
6
B. robur
hybrid
B. oblongifolia

3.2.2. Departure from formal null hypotheses

3.2.2.1. Hardy-Weinberg Equilibrium

Departures of genotype frequencies from those expected under Hardy-Weinberg equilibrium were determined using the total number of plants genotyped within the hybrid zone (shown in Figures 3.1 and 3.2), as well as the plants from the *B. oblongifolia* pure stands (the plants from the pure stand populations of *B. robur* were not used to determine departures from Hardy-Weinberg equilibrium, as all loci were monomorphic). Departures within the hybrid zones were calculated using BIOSYS-1 (Swofford and Selander 1981). Three pooled genotype classes were tested using a Chi-square analysis: the most common homozygote (the alleles of *B. robur*), heterozygotes and the least common homozygotes (the *B. oblongifolia* alleles).

Within the pure stand *B. oblongifolia*, the homozygote and heterozygote genotypes were pooled into two classes, to allow a valid Chi-square analysis. Departures of the observed frequency in each of these classes from that expected under Hardy-Weinberg equilibrium were tested for significance using Chi-square analysis of goodness of fit.

3.2.2.2. Linkage Disequilibrium

The presence of linkage disequilibrium between pairs of loci was determined using the method of Hill (1975) for a diallelic system. Ideally, the determination of the coefficient of linkage disequilibrium (D) should be done at regular intervals along the transect through the hybrid zone to determine the association of linkage with the position through the cline. However, within the *B. robur*/*B. oblongifolia* complex, the hybrid zone was too narrow to produce a series of sufficiently large sample size for the determination of D along the length of the transect. Estimates of the linkage disequilibrium coefficient were made for the pure stand populations of *B. oblongifolia*, and the hybrid zone populations. Chi-square analysis was used to determine the significance of D in each population.

As D is constrained by the allele frequencies (i.e. it is reduced, and so perhaps inaccurate, when the population is less polymorphic) (Szymura & Barton 1986), estimates of linkage between two loci were determined by the standardized pairwise linkage disequilibrium coefficient, R, from the relationship:

$$R_{ij} = D_{ij} / \sqrt{(pq_i pq_j)}$$

where D_{ij} is the coefficient of linkage disequilibrium between loci i and j, and pq_i and pq_j are the allele frequencies on loci i and j, respectively. As R_{ij} is essentially a correlation coefficient, its value lies between -1 and +1.

3.2.3. Cline Shape

3.2.3.1. Multi-locus Clines

To determine if the frequency of the *B. robur* alleles of the different loci change at the same rate along the cline, the frequency of the *B. robur* allele at each locus within each quadrat along the transect was plotted against its mean frequency across all loci, forming a cline for each locus. The deviations in these clines from the cline formed by the mean allele frequency (the y = x line), could be characterized by fitting a polynomial function to the curve relating the allele frequency at the locus (p_i) to the mean "*B. robur* allele" frequency (p) (Szymura & Barton 1986):

$$p_i = p + 2pq(\alpha + (p - q)\beta)$$

where α describes the shift of the individual locus cline from the mean towards the *B. oblongifolia* region, or the extent of introgression of the *B. robur* allele, and β describes the extent to which the individual locus cline is narrower than the mean cline, or the selective pressure on the individual locus.

3.2.3.2. Position of Cline

Comparison of the individual locus allele frequencies with the mean frequency of the *B. robur* allele over all loci is only an examination of similarity among clines formed by the individual loci within the one system, because the relationship does not use information on the shape of the clines (Szymura & Barton 1991). The frequency of the *B. robur* alleles at each locus, along with the mean of these allele frequencies, was plotted against the distance along the transect in each population, and polynomial functions were fitted to the resultant curves. The centre of the cline (y in Szymura & Barton 1986), was the position along the transect at which $p = 0.5$.

3.2.3.3. Cline Width

The cline width, w, was determined in two ways: 1. the inverse of the maximum slope of the cline (Endler 1977; Barton 1983), and 2. the geographic distance over which the allele frequency, or any other trait, changes from v to (1-v) (Endler 1977). The value of v is usually set at 0.2 (May *et al.* 1975), because these frequency values encompass the region of maximum slope of the cline. However, Bert & Harrison (1988) suggested that these boundaries should be modified for clines that do not reach pure species allele frequencies (i.e. p = 0 or 1), as a cline boundary set at 0.2 or 0.8 in this circumstance may actually encompass a portion of the "pure populations", or the cline tail. The new boundaries can be determined by:

$$f_1 = p_x + 0.2\Delta p$$

and

$$f_2 = p_x + 0.8\Delta p$$

where p_x is the frequency of the allele in the pure stands in which the allele is least common (i.e. in this study the frequency of the *B. robur* allele in the pure stand *B. oblongifolia*), and Δp is the difference in allele frequency covered in the step of the cline

(*d* in Bert & Harrison 1988). The cline width is then the geographic distance between f_1 and f_2

3.2.3.4. Introgression

The extent of introgression into the two parental populations can be estimated from the tail of the cline. Szymura and Barton (1986, 1991) have devised two parameters to describe the introgression within a population. The first, θ, is dimensionless and simply describes the rate of decay of the tail, providing an estimation of the amount of selection on the allele. Szymura and Barton (1986) described θ by the relationship:

$$e^{(-4x\sqrt{\theta}/w)}$$

but it is also equal to the square of the ratio between the scale over which the tail approaches 1 or 0, depending on the side of the cline, and the cline width. Since Slatkin (1973) shows that the characteristic distance over which the frequency of an allele changes (equivalent to the cline width) is inversely proportional to the square root of the selection pressure on that allele. θ, therefore, is also equal to the ratio of the selection acting against the introgression (or in the region outside the cline) of each marker allele (s_e) and the selection acting on the allele markers at the centre of the cline (s*), which is primarily due to linkage with other loci (Szymura and Barton 1986) (see section 3.2.4.).

The determination of θ is straightforward if an exponential expression is applicable to the tail. In the present study, however, an exponential function was appropriate only for the tail of the cline that approached y=0 (the *B. oblongifolia* end of the cline), while a linear expression better fitted the y=1 tail (the *B. robur* end). In fact, on the Darkes Forest cline and the northern section of the Cataract cline, the expression of best fit was y=1, indicating that there was no tail of introgression on the *B. robur* side of these clines. The linear expression on the southern Cataract cline formed a very short tail. The decision to fit different functions to the two different tails was based on several observations:

although in all cases the clines reached y=1, none reached y=0 within the confines of the transect used; this is supported by the presence of "*robur* alleles" in small frequencies in pure *oblongifolia* stands (Chapter Two), well removed from the mixed species stands, suggesting that y may never reach 0 in this system.

Any barrier to gene flow will produce a sharp step in the gene frequency, and the strength of this barrier will be reflected in the amount of introgression into the pure populations. The second parameter, B, measures this barrier to the flow of genes, and simply describes the total proportion of introgressing alleles within the pure populations. B is determined by the relationship:

$$B = \Delta p / p'$$

where Δp is the difference in allele frequency covered in the step of the cline, and p' is the slope (or maximum slope in the case of the exponential function) of the tail. This parameter has the dimensions of length and, therefore, indicates the tail length. This expression was used to determine B_o (the distance of gene flow along the *B. oblongifolia* tail). The lack of a tail on the *B. robur* side of the clines meant that an estimate for B_r could not be made for the the Darkes Forest and the northern Cataract clines, but was estimated for the Cataract southern cline by simply determining the length of the tail when the line intercepted y=1.

3.2.4. Dispersal and selection parameters determined from cline shape

The use of the cline shape parameters (w and θ) and linkage disequilibrium (R) can be used to determine dispersal rate of parental genes and the strength of selection of the organisms within a hybrid zone.

If the cline is maintained through dispersal of parental genes into the hybrid zone and the selection against hybrids, the association between loci will be strongest in the centre, and

weaker towards the edges of the cline, as the genomes at the edge will be introgressed, and therefore will have had more generations to recombine and lose the association (Szymura & Barton 1986). In the centre of the cline, the dispersal can be determined using the standardized linkage disequilibrium coefficient, R, using the expression

$$R = 4 \sigma^2 / w^2 r$$

where σ^2 is the dispersal rate (in metres generation^{-1}), w is the width of the cline, and r is the recombination rate (which equals 0.5 in unlinked loci) (Szymura & Barton 1991). This method is approximate, as some linkage may not be solely due to dispersal, although the effects of the other causes of disequilibrium will be small as the number of loci observed increases or the selection decreases (Barton 1983).

The estimated rate of dispersal can be used as a prediction of the dispersal of pollen within the population assessed through other means, such as observations of pollinator activity and by following the pattern of rare alleles within the population (addressed in Chapter Five) (seed dispersal in *Banksia* is thought to be negligible [Abbott 1985]). The dispersal rate must be interpreted cautiously when considering dispersal within organisms with overlapping generations, such as *Banksia*. These calculations assume generations are fairly well defined (Szymura & Barton 1986). Within *Banksia*, on the other hand, mating can occur between generations, and the products of these matings are accumulated within a plant, and released at irregular intervals (mostly after fire). This means that the progeny of perhaps years of mating are released simultaneously as a cohort. However, the pattern of genotypes within the population will only alter as a result of the release of the cohort of seed after fire. This is the time frame, therefore, that will be used for dispersal rate within this study (metres cohort^{-1}). These definitions will also be used in Chapter Five.

Two measures of selection can now be calculated from the estimate of dispersal and the cline shape parameters. The effective selection acting on each individual enzyme locus or the selection pressure maintaining the cline (s*), can be determined from the dispersal and cline width (Barton 1983, Szymura & Barton 1986):

$$s^* = \sqrt{8}\ \sigma^2 / w.$$

In a cline maintained by a balance between selection and dispersal, w is equivalent to the characteristic scale of selection (Slatkin 1973). This relationship is similar to the method for determining the magnitude of selection acting against the hybrids (s) (Barton & Hewitt 1985, Bert & Harrison 1988), which is proportional to the square of the ratio between the dispersal (σ^2) and the cline width (w).

Using s*, the selection on introgressing marker alleles (s_e) can be determined from θ, as mentioned above under Section 3.2.4.4., using the relationship

$$\theta = s_e / s^*$$

(Szymura & Barton 1991).

3.2.5. Plant vigour and fecundity

Each plant was categorized as either *B. robur*, hybrid or *B. oblongifolia*, using their scores on both the genetic and the morphological hybrid indices, devised in Chapter Two. Both indices were used to place the plants in each phenotypic class, because many plants within the hybrid zones lacked sufficient appropriate tissue for electrophoresis. To assess the survivorship, vigour and fecundity of each plant, four key characters were measured: the proportion of *B. robur*, *B. oblongifolia* and hybrid plants within each quadrat, plant height, the total number of inflorescences produced and the mean number of follicles per infructescence. These four characters were used to summarise the fitness of each plant.

The proportion of each phenotypic class in each quadrat may reflect the deficit or excess of hybrids or parental types along the transect. Plant height is often measured to determine plant vigour, as it reflects physiological fitness through increased plant biomass. As all plants within the hybrid zone were probably razed by fire at the same time, differences in plant height will also indicate increased growth rate, necessary for competition with the faster-growing dominant sedges. The fecundity of the plant was indicated by the number of inflorescences per plant and the number of follicles produced per infructescence. It was hoped that these measurements would reflect both the paternal (proportion of inflorescences available as pollen donators) and the maternal (number of seed formed by each infructescence) fitness or ability of the plants. Differences between the categories in plant height, number of inflorescences per plant and the number of follicles per infructescence were detected using One-way Analyses of Variance (Model I). When significant heterogeneity was detected between groups using the ANOVA, a Tukey test was employed to determine which groups contributed to the significant differences (Zar 1984).

The proportion of the phenotypic classes and the mean of each of plant height, total number of inflorescences and the number of follicles per infructescences were determined for each of the three plant types within the quadrats used to determine the allele frequency clines along the transects in each site, above. These values were then plotted against the distance along the transect.

3.3. Results

3.3.1. Spatial pattern of genotypes within the hybrid zones

The hybrid zones in both populations contained genotypically diverse hybrid swarms (Figures 3.1 and 3.2), showing complex spatial patterns and intermixing of plants with parental and hybrid origin genotypes.

The one-dimensional representation of the plants within each hybrid zone confirmed the complex spatial arrangement of genotypes. Along each transect in both the Cataract and Darkes Forest sites, there were frequent transitions from high frequencies of one parental type to the other within very short distances. In both sites, the frequency of the *B. robur* allele reached or approached unity in more than one region along the transect. The transect through the Cataract population has a region of intermediate allele frequencies, bounded by two *B. robur* regions (Figure 3.3). Along the transect through the Darkes Forest population (Figure 3.4) transect there is a short region where the *B. robur* allele frequency approaches zero (the level at which these alleles are found in pure *B. oblongifolia* stands).

3.3.2. Departures from formal null hypotheses

3.3.2.1. Hardy-Weinberg Equilibrium

There were heterozygote or hybrid deficits at all loci in both hybrid zones. Only one of these was not significantly different from the proportions expected under Hardy-Weinberg equilibrium: the *Adh* genotypes within the Cataract hybrid zone (Table 3.1b). In contrast, both populations of pure stand *B. oblongifolia* generally showed no significant difference between the observed proportions and those expected under Hardy-Weinberg equilibrium. One exception, the *Gdh* locus in the Cataract population, showed a significant excess of heterozygotes (Table 3.2a).

3.3.2.2. Linkage Disequilibrium

Significant linkage disequilibrium was detected only for pairwise comparisons within the hybrid zone (Table 3.3c & d). There was no evidence of linkage disequilibrium within the pure stand of *B. oblongifolia* (Table 3.3a & b). No tests were possible within the invariant pure stands of *B. robur*.

Figure 3.3. The genetic cline along the Cataract transect. (a) The change in frequency of *B. robur* alleles at three loci along the transect. The frequency of each allele was determined within each of 13 quadrats. The error bars are the sampling errors (b) The mean *B. robur* allele frequency along the transect. (c) Plant sample sizes within each quadrat along the transect.

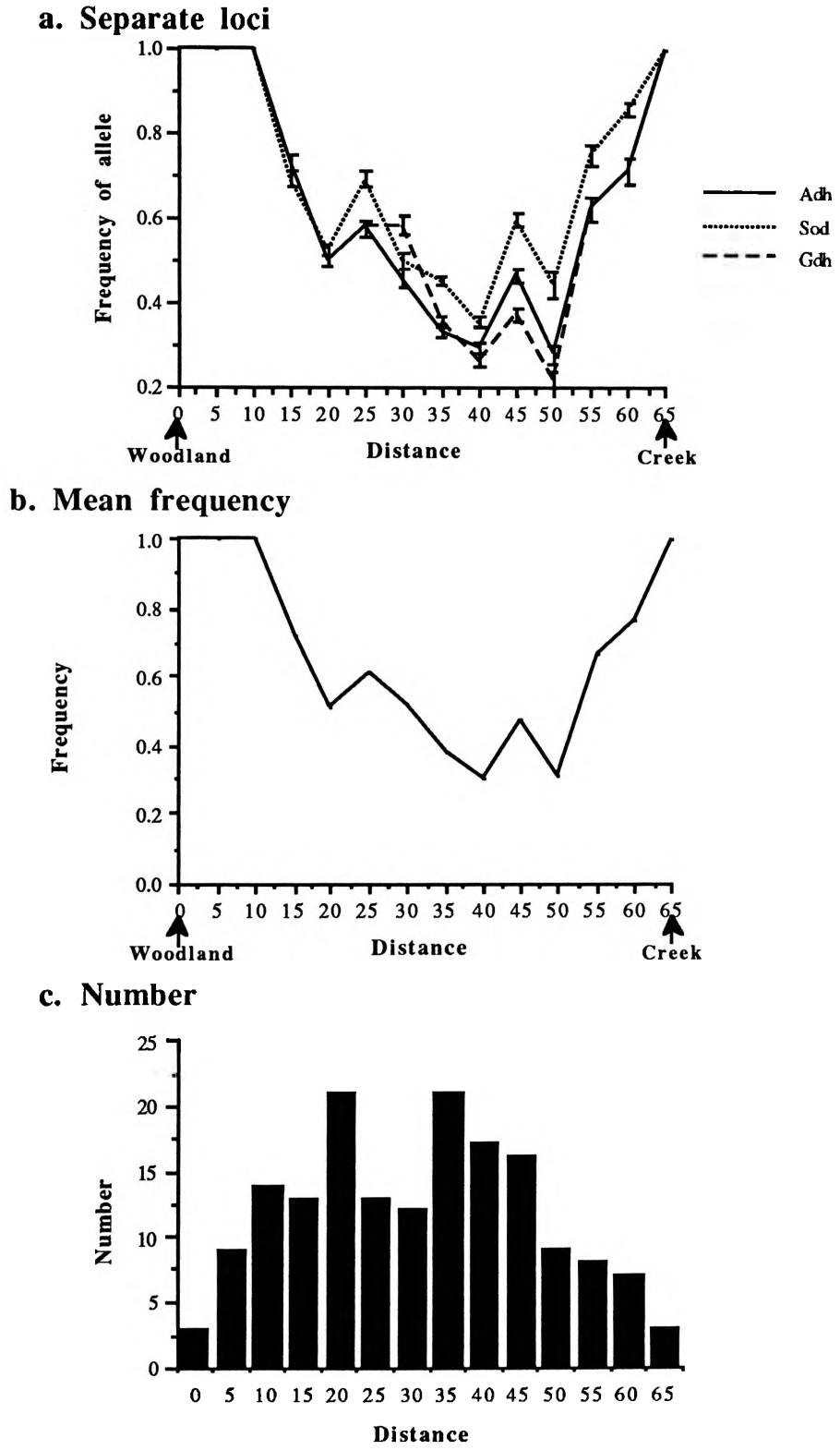


Figure 3.4. The genetic cline along the Darkes Forest north-south transect (comprising of 16 quadrats).

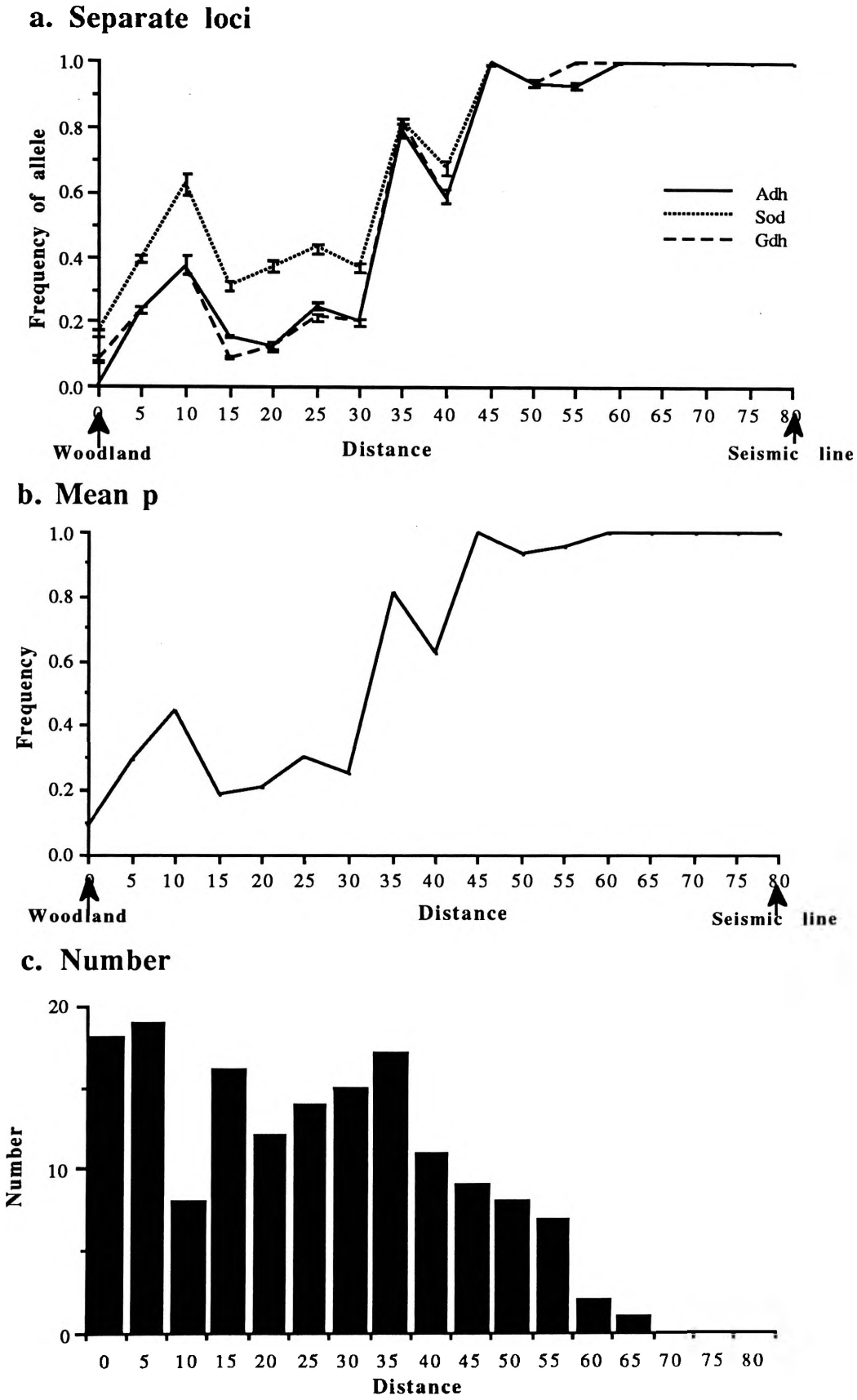


Table 3.1. Deviations from genotype frequencies expected under Hardy-Weinberg equilibrium in two pure *B. oblongifolia* populations within three loci. Classes tested were pooled homozygotes and heterozygotes. The first figure is the observed frequency and the figure in brackets is the expected frequency under each class for each locus. The asterisk indicates the level of significance of the deviation is $p < 0.05$. NS indicates the deviation is not significant. - under classes indicates that there was no variation at that locus, and therefore a test could not be performed.

	Heterozygotes	Homozygotes	
a. Darkes Forest			
Adh	3 (2.85)	18 (18.15)	NS
Sod	8 (6.63)	13 (14.37)	NS
Gdh	-	-	
b. Cataract			
Adh	-	-	
Sod	13 (9.69)	2 (5.31)	NS
Gdh	14 (9.38)	1 (5.62)	*

Table 3.2. Deviations from genotype frequencies expected under Hardy-Weinberg equilibrium in two hybrid zone *B. oblongifolia* populations within three loci. Classes tested were the "*B. robur* allele" homozygotes, hybrid heterozygotes and *B. oblongifolia* homozygotes. The first figure is the observed frequency and the figure in brackets is the expected proportion under each class for each locus. The asterisks indicate the level of significance of the deviation: * < 0.05; ** < 0.01; *** < 0.001.

	B. robur	Hybrid	*B. oblongifolia*	
a. Darkes Forest Hybrid Zone				
Adh	31 (26.4)	35 (44.4)	23 (18.3)	*
Sod	38 (32.6)	32 (42.7)	19 (13.6)	*
Gdh	32 (25.2)	31 (44.6)	26 (19.2)	**
b. Cataract Hybrid Zone				
Adh	66 (63.1)	52 (57.9)	16 (13.1)	NS
Sod	69 (58.3)	39 (60.3)	26 (15.3)	***
Gdh	64 (56.4)	46 (61.3)	24 (16.4)	**

Table 3.3. Estimates of the coefficient of linkage disequilibrium (D) determined using the method of Hill (1975). The two hybrid zone populations consisted of a mixed sample of plants within the hybrid zone (i.e. *B. robur*, hybrids and *B. oblongifolia*), while the pure stand populations were the pure stand *B. oblongifolia* only. The sample size in each population is denoted n. * indicates significant linkage disequilibrium between loci at $p<0.001$. - under D indicates that loci tested were invariant within that population.

Loci	D
a. Darkes Forest Pure Stand: n=21	
Adh / Sod	0.00
Adh / Gdh	-
Sod / Gdh	-
b. Cataract Pure Stand: n=15	
Adh / Sod	-
Adh / Gdh	-
Sod / Gdh	0.03
c. Darkes Forest Hybrid Zone: n=89	
Adh / Sod	0.21*
Adh / Gdh	0.25*
Sod / Gdh	0.21*
d. Cataract Hybrid Zone: n=134	
Adh / Sod	0.21*
Adh / Gdh	0.22*
Sod / Gdh	0.25*

Both hybrid zone populations had mean standardized linkage coefficients (R) at or close to +1, indicating that all loci sampled were effectively totally linked in the hybrid zone (Table 3.4).

3.3.3. Cline Shape

3.3.3.1. Coincidence of individual loci

The three loci varied a little, but showed similar patterns of clinal variation. In particular, the *Adh* and *Gdh* loci clines corresponded closely to each other, and were steeper than the *Sod* cline along both transects surveyed (Figure 3.5a & b). Sampling errors within each quadrat are relatively small (Shown in Figures 3.3a and 3.4a), indicating that the difference between the *Sod* and the other two clines is real, and not because of error noise.

The parameters calculated for each cline, describing the shift in cline position (α) and the change in cline width (β), reinforced some of the similarities in the position of the clines (Table 3.5). For example, the graphical coincidence of the clines formed by *Adh* and *Gdh* on the Darkes Forest transect (Figure 3.5b) is confirmed by the similarities of the position and width of the cline, indicated by the magnitude and sign of the values of α and β (Table 3.5b).

The values of α obtained for the *Adh* and *Gdh* transects in both sites were negative, although small in magnitude. This indicates that, at these loci, there is a decrease in *robur* alleles below the average, and therefore a shift, albeit slight, towards *B. robur* territory. The positive values of α shown by both *Sod* transects are greater in magnitude than the α values obtained for *Adh* and *Gdh*, suggesting a shift in the position of the cline towards *oblongifolia* territory. The *Sod* cline in the Darkes Forest site shows an increase in the cline width (negative β), indicating an increase of *robur* alleles on the *oblongifolia* side of the cline. All other locus clines show a decrease in width.

Table 3.4 Estimates of standardized pairwise linkage disequilibrium coefficient, R_{ij}, within the hybrid zone populations. The mean R_{ij} over all pairs of loci in both populations is given with its standard error.

		Darkes Forest		
		Adh	***Sod***	***Gdh***
Cataract	***Adh***	-	0·868	1.001
	Sod	0.951	-	0.867
	Gdh	0.994	1.100	-
	Mean	= 0.914 ±0.038		

Figure 3.5. Comparison of the clines formed by the different loci, determined from the plot of frequency of the *B. robur* allele at each locus (Frequency p) versus the mean allele frequency (Mean p) within each quadrat along the a. Cataract transect. b. Darkes Forest transect. The function of best fit of each locus cline is used to determine α and β (see Table 3.5.).

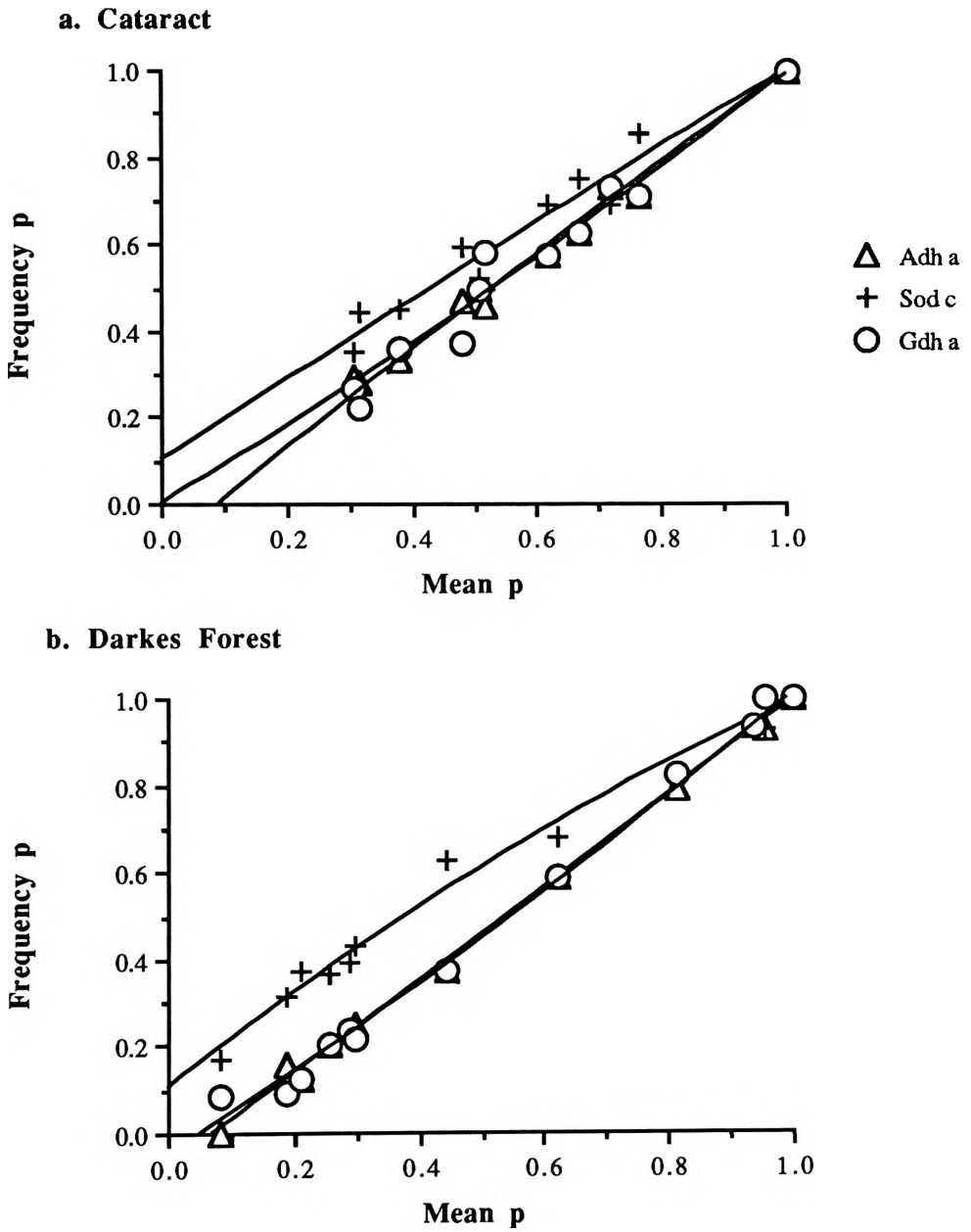


Table 3.5. Estimates of α and β for each locus, on each cline along the transects at a. the Cataract site and b. the Darkes Forest site. α describes the shift of the individual locus from the mean toward the *B. oblongifolia* region, while β shows the extent to which the individual locus is narrower than the mean cline.

		Adh	*Sod*	*Gdh*
a. Cataract				
	α	-0.063	0.124	-0.061
	β	0.003	0.046	0.086
b. Darkes Forest				
	α	-0.088	0.223	-0.109
	β	0.058	-0.103	0.052

3.3.3.2. Cline position and width

In order to simplify the calculation of the cline shape parameters for each hybrid zone, best-fit curves were fitted to each of the graphs, formed by the transition in allele frequency along the transects (Figures 3.6 and 3.7). In the Cataract site, the transect traversed from regions of high, through intermediate, and again to high *robur* allele frequency. Along this transect, therefore, two clines were fitted: the north cline (high to low *robur* allele frequency) and south (low to high *robur* allele frequency) (Figure 3.6).

The values of *w* obtained from the two methods were fairly different, and there were substantial differences between the sites (Table 3.6). It is difficult to assess which is the most accurate measurement, as both methods may be appropriate in different situations. The inverse of the maximum slope may underestimate the width of the cline, particularly if the cline does not increase smoothly. Taking the geographic distance between the regions where the frequency of a trait changes from v to (1 - v), however, ignores the changes that have occurred within these boundaries. In this study, the inverse of the maximum slope was used in all parameters requiring an estimate of the cline width, as it was judged in this study that the cline width was greatly underestimated using the geographic distance between the change in frequency from f_1 to f_2, especially at the end of the cline approaching p=1.

3.3.3.3. Introgression

The rate of decay of the *B. oblongifolia* cline tail (θ_o) was determined directly from the fitted exponential expression. The results indicate that the rate of decay of all the tails is slow ($\theta_o << 1$) (Table 3.6) The estimated distance of gene flow along the *B. oblongifolia* tails (B_o) indicated that there was little barrier to *robur* alleles in the *oblongifolia* genome, but essentially no infiltration of *B. oblongifolia* alleles into the *B. robur* genome.

Figure 3.6. The curves fitted to the transect of the Cataract site. The transect is divided into two clines, the north cline (on the left) and the south cline (on the right). Straight lines were fitted to the *B. robur* tails (as the allele frequency approaches 1) and exponential curves to the *B. oblongifolia* tails (as y approaches 0). The vertical lines correspond to the centre of each cline, where y = 0.5.

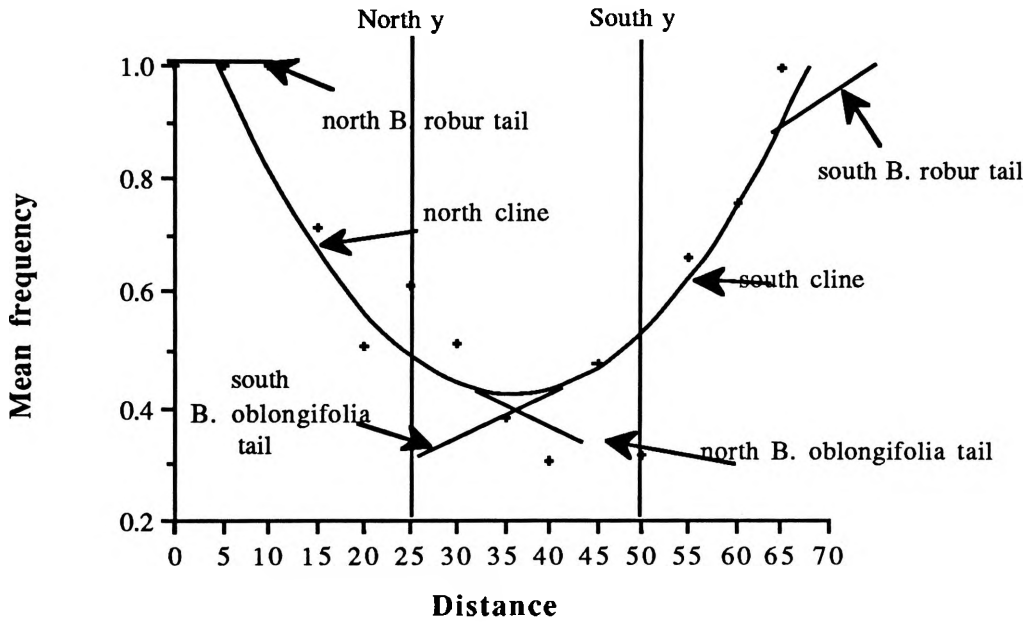


Figure 3.7. The cline along the transect of the Darkes Forest site. A line (y=1) was fitted to the *B. robur* tail, while an exponential curve was fitted to the *B. oblongifolia* tail.

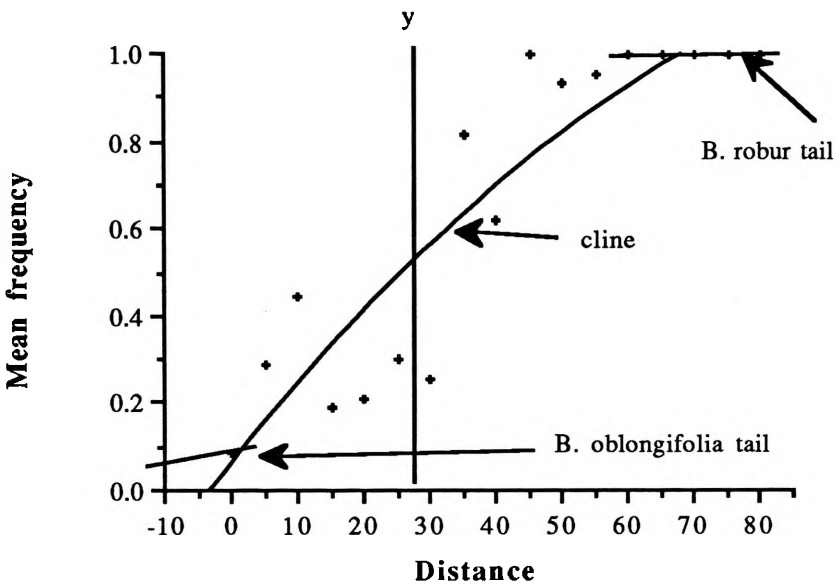


Table 3.6. Estimates of the parameters determining shape of cline. The cline width (w) is estimated using two methods: a. the inverse of the maximum slope (Endler 1977) and b. The geographic distance between the areas that have the frequency f_1 and f_2 of a certain trait (Bert & Harrison 1988). θ_o is the rate of decay of the tail of introgression on the *B. oblongifolia* side of the cline (θ_r was not able to be estimated) and B is the barrier to gene flow. Both w and B have the dimension of length (in metres), while θ is dimensionless.

Cline	w		θ_o	B_r	B_o
	a	b			
Darkes Forest	62.5	41.48	0.0369	0	173
Cataract (North)	27.8	42.24	0.0085	0	80
Cataract (South)	34.48	24.39	0.0299	3.33	63.83

3.3.4. Dispersal and selection

There was variation between populations and clines in the estimates of dispersal rate (Table 3.7). For Darkes Forest, σ^2 was in the order of 0.5 kilometres, while the estimates of the dispersal rate for the Cataract catchment were no more than a third of the estimate for Darkes Forest. These dispersal rates were approximately five times that of the width of the clines in each case.

The two measurements of selection determined for these hybrid zones showed that there were different selective regimes on the alleles within and outside the hybrid zone. The effective selection pressure (s*), which indicates the pressure on the *B. robur* allele in the centre of the cline, was extremely high (at or almost 100%) (Table 3.7). This magnitude of this parameter largely reflects the level of linkage disequilibria in the centre of the cline. The selective pressure on these alleles outside the cline, (s_e) was very low (Table 3.7).

3.3.5. Plant vigour and fecundity

In the Cataract site, only plant height varied significantly between the groupings of *B. robur*, hybrids and *B. oblongifolia* based on GHISs (Table 3.8a). *B. robur* plants were on average about 25% larger than the *B. oblongifolia* plants (a Tukey test, Table 3.8b). The group of hybrid plants were significantly different from the *B. oblongifolia* group, but were not significantly different from the *B. robur* group.

In the Darkes Forest population, significant differences were detected between *B. robur*, hybrid and *B. oblongifolia* groupings in the number of inflorescences per plant only (Table 3.9a). The *B. oblongifolia* group had significantly larger numbers of inflorescences per plant than the hybrid group (a Tukey test, Table 3.9b). The number of inflorescences per plant within the *B. robur* group did not vary significantly with the *B. oblongifolia* group, nor the hybrid group.

Table 3.7. Estimates of dispersal rate (σ^2, metres / cohort), effective selective pressure on loci within the cline (s*) and the selective pressure on the loci outside the cline (s_e). Separate estimates are given for the Darkes Forest cline, and the north and south Cataract clines.

	σ^2	s*	s_e
Darkes Forest	446.05	0.96	0.035
Cataract (north)	98.54	1.01	0.009
Cataract (south)	151.58	1.01	0.030

Table 3.8. a.One-factor ANOVA for each character, measured from plants within the Cataract site. The levels within the factor are the three groupings of plants categorized *B. robur*, hybrid and *B. oblongifolia* groupings, using the genetic hybrid index, determined in Chapter 2. DF - degrees of freedom, F - F statistic and P - probability. NS indicates that no significant difference was detected (P>0.05); * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001. b. Characters measured from each plant in the Cataract site, categorized using the genetic hybrid index. R, H and O are the *B. robur*, hybrid and *B. oblongifolia* plants, respectively. The were plants categorized into these three groups using the cut off points described in Chapter 2. The figures show the mean of the group for each character (with the standard error in brackets. The black bars show the result of the Tukey test performed on the means based on the results obtained in the one-way ANOVA, and join the plant groupings not significant different (p>0.05). Sample sizes are: for characters a and b; R = 59, H = 43, O = 20. Character c; R = 44, H = 30, O = 13.

a.

Character	Source of Variation	DF	Mean Square	F	P
a. Height	**Groups**	2	4589.71	5.54	**
	Within Groups	119	828.29		
b.Inflorescences per plant	**Groups**	2	43.27	0.59	NS
	Within Groups	119	73.6		
c.Follicles per infructescence	**Groups**	2	102.98	0.77	NS
	Within Groups	84	133.69		

b.

a.	Height	R	H	O
		117.9	113.7	93.3
		(3.42)	(5.34)	(4.31)
b.	Inflorescences per plant	H	O	R
		11.3	10.1	9.8
		(1.60)	(2.27)	(0.80)
c.	Follicles per infructescence	O	R	H
		31.7	28.6	27.0
		(3.72)	(1.79)	(1.87)

Table 3.9 a. One-factor ANOVA for each character, measured from plants within the Darkes Forest site. The levels within the factor are the three groupings of plants categorized *B. robur*, hybrid and *B. oblongifolia* groupings, using the genetic hybrid index, determined in Chapter Two. DF - degrees of freedom, F - F statistic and P - probability. NS indicates that no significant difference was detected (P>0.05); * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001. b. As for Table 3.8b, but plants are from the Darkes Forest site. Sample sizes are: for characters a and b; R = 21, H = 32, O = 21. Character c; R = 13, H = 27, O = 18.

a.

Character	Source of Variation	DF	Mean Square	F	P
a. Height	Groups	2	102.7	0.21	NS
	Within Groups	71	487.6		
b.Inflorescences per plant	Groups	2	1315.0	5.42	**
	Within Groups	71	242.5		
c.Follicles per infructescence	Groups	2	637.1	2.58	NS
	Within Groups	55	247.0		

b.

a.	Height	H	R	O
		116.4	114.8	112.4
		(4.58)	(3.85)	(4.26)
b.	Inflorescences per plant	O	R	H
		24.7	13.7	10.6
		(4.98)	(3.24)	(1.54)
c.	Follicles per infructescence	H	R	O
		36.8	31.2	26.0
		(3.42)	(4.04)	(3.05)

When all plants at each site was considered (grouped using the morphological hybrid index), the 1-way ANOVA revealed that more characters were significantly different between plant groups. In the Cataract population, significant differences were detected between the groups in plant height and number of inflorescences per plant (Table 3.10a). *B. oblongifolia* plants were significantly shorter than both other groups, while there were significantly greater inflorescences per hybrid plant than both other groups (a Tukey test, Table 3.10b.).

In the Darkes Forest site, significant differences were detected between the plant groups in only the number of inflorescences per plant (Table 3.11a). *B. oblongifolia* plants had significantly more inflorescences per plant than both the hybrid and *B. robur* plants (Table 3.11b).

There was a smaller proportion of hybrids than either of the parental groupings within each quadrat along each of the transects and there was no consistent spatial pattern in the vigour and fecundity characters (Figures 3.8 and 3.9). The hybrid plants are not consistently larger than either of the parent species in the regions along the transect corresponding to the allele frequency cline, nor are they consistently smaller or less fecund.

3.4. Discussion

The results described in this Chapter provide conflicting evidence as to the type of hybrid zone that has been formed by *Banksia robur* and *Banksia oblongifolia.* There is evidence that the clines are formed as a result of adaptation to the environment (as defined by Endler 1977), or independently of the environment, and is maintained through selection of hybrid progeny and dispersal of parental genotypes into the hybrid zone (as defined by Barton & Hewitt 1981, 1985). The heterogeneity of the distribution of the genotypes within the sites, and the close association of *B. robur* with water courses or water-logged soil and *B. oblongifolia* with the better drainage of the surrounding woodlands, suggest

Table 3.10. One-factor ANOVA for each character, measured from plants within the Cataract site. The levels within the factor are the three groupings of plants categorized *B. robur*, hybrid and *B. oblongifolia* groupings, using the morphological hybrid index, determined in Chapter 2. DF - degrees of freedom, F - F statistic and P - probability. NS indicates that no significant difference was detected (P>0.05); * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001. b. As for Table 3.8b, but plants categorized using morphological index. Sample sizes are: for characters a and b; R = 175, H = 38, O = 86. Character c; R = 108, H = 24, O = 47.

a.

Character	Source of Variation	DF	Mean Square	F	P
a. Height	**Groups**	2	19029.6	25.57	****
	Within Groups	296	744.15		
b.Inflorescences per plant	**Groups**	2	275.2	5.25	**
	Within Groups	296	52.5		
c.Follicles per infructescence	**Groups**	2	353.3	2.23	NS
	Within Groups	176	158.7		

b.

a.	Height	H	R	O
		122.4	113.0	90.6
		(6.24)	(2.15)	(2.15)
b.	Inflorescences per plant	H	R	O
		11.5	7.9	7.1
		(1.79)	(0.46)	(0.78)
c.	Follicles per infructescence	O	R	H
		28.4	24.9	22.1
		(1.80)	(1.29)	(1.77)

Table 3.11. One-factor ANOVA for each character, measured from plants within the Darkes Forest site. The levels within the factor are the three groupings of plants categorized *B. robur*, hybrid and *B. oblongifolia* groupings, using the morphological hybrid index, determined in Chapter 2. DF - degrees of freedom, F - F statistic and P - probability. NS indicates that no significant difference was detected (P>0.05); * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001. b. As for Table 3.9, categorized using the morphological index. Sample sizes are: for characters a and b; R = 57, H = 29, O = 78. Character c; R = 28, H = 18, O = 62

a.

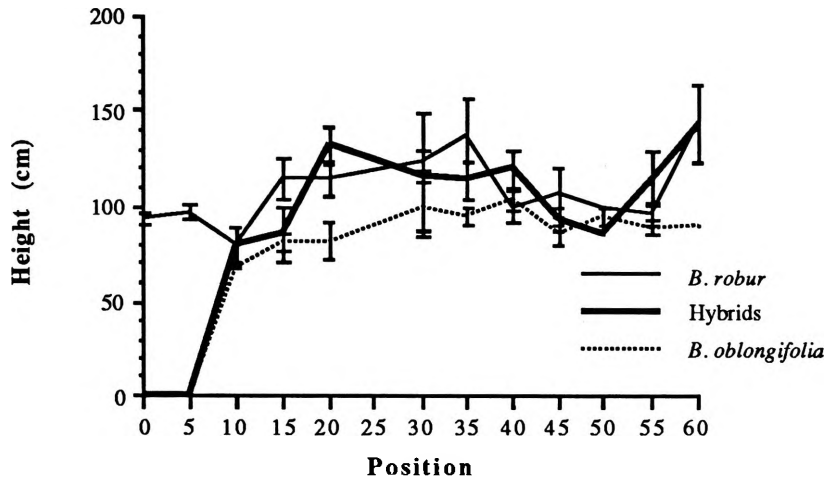
Character	Source of Variation	DF	Mean Square	F	P
a. Height	**Groups**	2	519.7	1.05	NS
	Within Groups	161	496.6		
b.Inflorescences per plant	**Groups**	2	1949.9	12.21	****
	Within Groups	161	159.7		
c.Follicles per infructescence	**Groups**	2	278.0	1.09	NS
	Within Groups	105	256.3		

b.

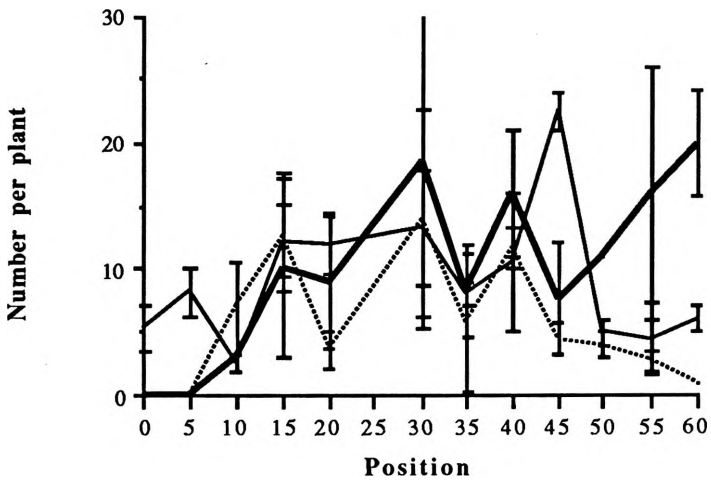
a.	Height	H	O	R
		113.3	109.0	106.0
		(5.21)	(2.39)	(2.72)
b.	Inflorescences per plant	O	R	H
		17.6	8.4	6.9
		(1.70)	(1.42)	(1.55)
c.	Follicles per infructescence	H	R	O
		32.9	29.4	26.8
		(4.56)	(3.50)	(1.73)

Figure 3.8. The change in the mean of a. plant height. b. number of inflorescences per plant. c. number of follicles per infructescence, each group of plants (i.e. *B. robur*, hybrid and *B. oblongifolia*) along the Cataract transect.

a. Plant height



b. Inflorescences per plant



c. Follicles per infructescence

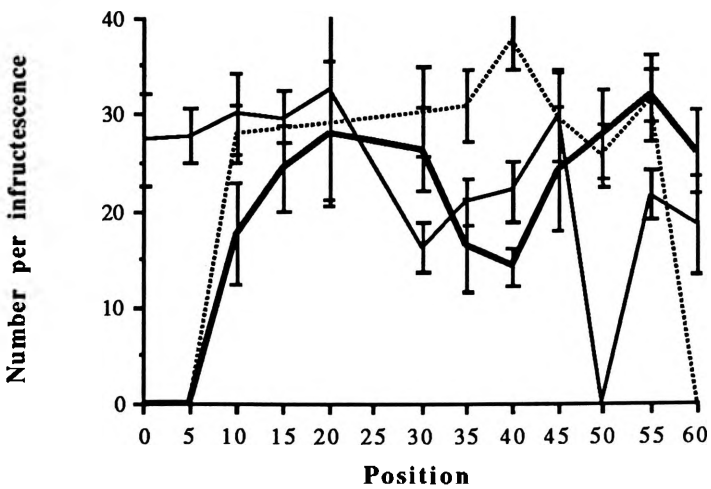
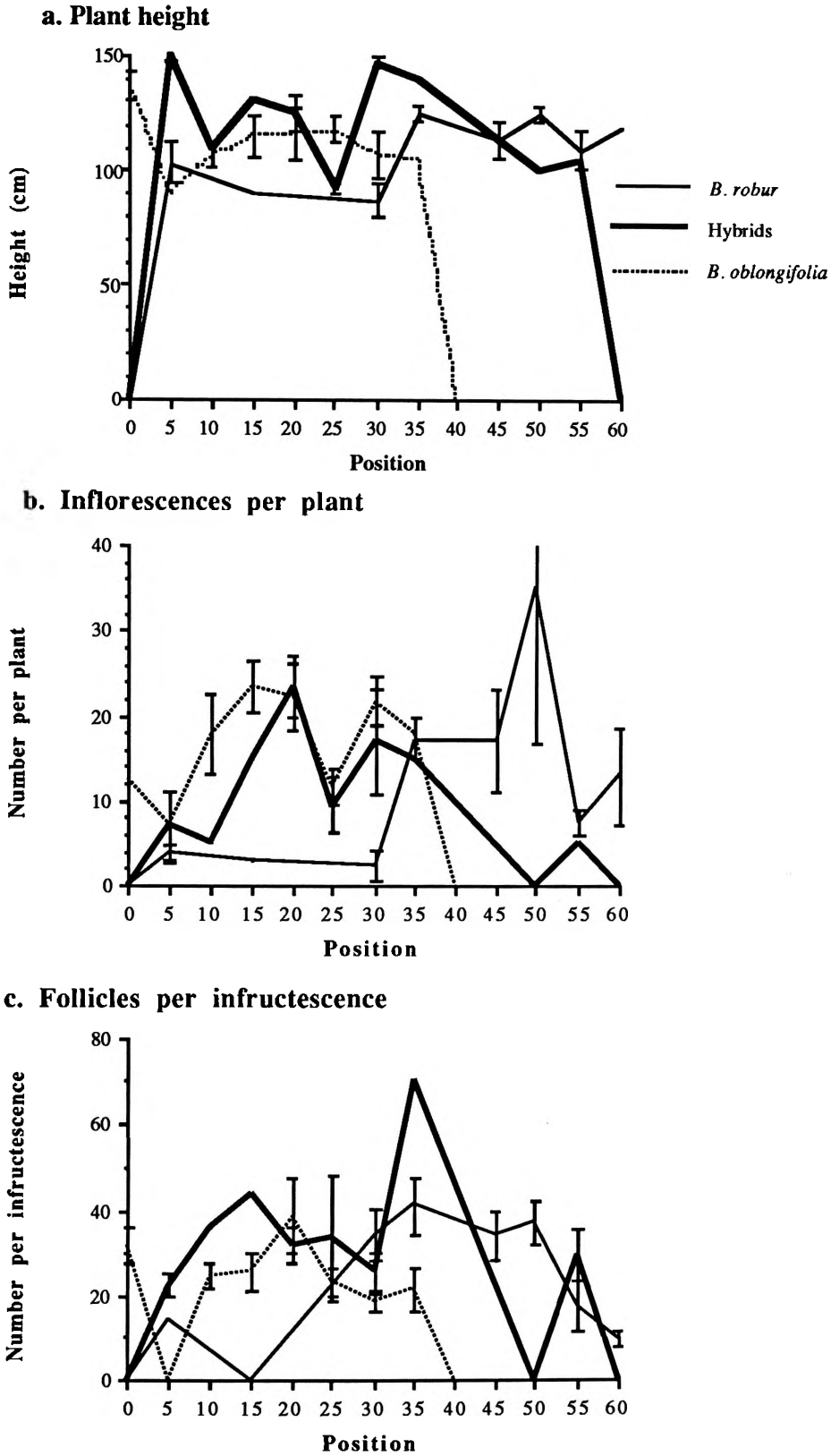


Figure 3.9. As for Figure 3.8, but for the Darkes Forest transect.



that the parental genotypes are tightly linked to the environment. On the other hand, seemingly high selection against hybrid genotypes within the hybrid zone suggests hybrid disadvantage.

The application of this theory to the hybrid zone formed by *B. robur* and *B. oblongifolia* may not be entirely suited, primarily because of the narrowness of the hybrid zone and the limited sample size of plants across the transect. This would mainly affect the accuracy of the cline, thereby making the estimate of the cline shape parameters inaccurate. However, the results obtained in this Chapter do provide an indication of the selection acting on the hybrids within the hybrid zone, the dispersal occurring within the system, providing expectations on these aspects used in following sections of the study. As far as I am aware, this is also the first application of this model to a plant system.

3.4.1. Genotypic patterns within the *B.robur*/ *B. oblongifolia* hybrid zone

The spatial distribution of the different types of plants within each site did not form a simple gradation from one species through a hybrid zone into the other species, but a fairly complex mixing of genotype classes within the zone. This complexity observed within the hybrid zones may be the result of one of two things. Firstly, the sites were observed at a fairly high resolution, which may result in a higher level of heterogeneity or patchiness detected in the arrangement of the plant types. Secondly, the different classes of genotypes may be associated closely with particular environmental conditions, which are often found in small spatial areas, no larger than the canopy or the root ball of the plant that occupy them (Tilman 1982).

The dependence of establishment of different genotypes on particular micro-environments is also suggested by the geographic distribution of the species. If the two species form what has been described by Harrison (1986) as a mosaic hybrid zone, adaptation to the environment may enable hybrid individuals to establish and maintain the hybrid zone.

3.4.2. Cline Shape

The clines described in this study are very narrow, and are much narrower than the width predicted for this system in Section 3.1.2.2. Clines less than 100 metres are rarely described for animals (for reviews see Endler 1977, Barton & Hewitt 1985), although studies seem to be largely restricted to the more "mobile" organisms, such as insects, birds and mammals. Analyses of clines formed by animals that are sessile or possess a limited dispersal capability are needed. Narrow clines in hybrid zones of plants are more common (e.g. Heywood 1986, Arnold *et al.* 1992). The narrowness of plant clines may reflect restricted gene flow, as most plants are dependent on outside factors for pollen and seed movement. Except for some of the grasses, the cline widths recorded for the *B. robur* and *B. oblongifolia* hybrid zones are among the narrowest reported amongst plants (see Endler 1977).

Essentially, the cline width is a function of the pattern of the dispersal of the species involved, the time (number of generations) since contact (in the case of secondary intergradation) and the selection forces acting on the population (Endler 1977). At least one of these parameters is needed, along with the cline width, to determine the other two. The cline width in the context of the hybrid zone, plus the zones of introgression can also indicate the extent of gene flow occurring between the species, and has been used in this study in the calculation of the rate of decay of the tails into the *B. oblongifolia* regions of each cline.

The decay of the *robur* alleles in the *oblongifolia* genome appears to be very slow. This is confirmed by the genetic analysis done in Chapter Two, which showed the presence of "*robur*" alleles in the pure *B. oblongifolia* stands in substantial amounts. In contrast, the frequency clines indicate that there was no introgression of the *B. oblongifolia* alleles into the *B. robur* genome, and this is confirmed by the lack of variability in *B. robur* plants in the mixed and pure stands. This seemingly total barrier to the flow of the *B. oblongifolia* alleles into the *B. robur* areas suggests there is asymmetry in the gene flow in this

complex, a result not uncommon in hybrid zones (e.g. in *Mus* (Hunt & Selander 1973) and *Gryllus* (Harrison 1986)). This asymmetry may reflect the lack of opportunity for gene exchange in one direction at some stage of the life-cycle: differences in flowering phenology, pollen vector preferences or assortative mating. These aspects of the mating system within the hybrid zone will be investigated in subsequent Chapters of this thesis.

3.4.3. Multilocus effects

The clines of each locus corresponded closely, but not perfectly. There is a slight, probably significant shift of the *Sod* cline towards the *B. oblongifolia* side of the cline, indicating that Sod^S may decay more slowly than the other alleles towards *B. oblongifolia*. This is also suggested by the frequency of Sod^S in the pure populations of *B. oblongifolia* (Table 2.2), where it is present in a greater proportion than the "*B. robur* alleles" on the *Adh* and *Gdh* loci.

While there was some deviation in the clines formed by each locus from the cline of the mean allele frequency, the values for both α and β obtained for the *Adh* and *Gdh* clines in this study were comparable to, or less than, the results given by Szymura and Barton (1986) for *Bombina*. They concluded that the clines formed by the loci were essentially concordant, and that all the loci are under the same level of selection. The most likely scenario is that the allozymes are selectively neutral (Barton & Hewitt 1983). This conclusion does not weaken the hypothesis that this hybrid zone is formed as a result of the environmental differentiation. The loci surveyed are a tiny fraction of the genome and other genes not detected may be favoured in certain environmental conditions.

It is usual that when many clines coincide, linkage disequilibria will be particularly strong within the centre of the cline, fed by the continual dispersal of parental genotypes into, and the selective disadvantage of hybrid combinations within, the centre of the cline (Barton & Hewitt 1985). This situation has also been found in this study. The strong linkage disequilibrium between all loci sampled within the *B. robur/B.oblongifolia* hybrid

zones is typical of many other clines (e.g. Kocher & Sage 1986, Szymura & Barton 1986, Howard & Waring 1991). Further, the lack of association between loci in the pure populations of *B. oblongifolia* suggests that the linkage disequilibrium in the centre of the cline is maintained through the dispersal of parental pollen into the hybrid zone.

Another effect of coincident clines is the strengthening of the barrier to gene flow (Barton 1983). As the separate clines approach the diagonal (or the mean cline), β also approaches zero (characteristic of a narrowing of the cline), resulting, therefore, in less opportunity for recombination. This may explain the strength of the barrier to *B. oblongifolia* alleles into *B. robur*, and therefore the near purity of the *B. robur* genome, which was concluded from the analysis of the cline tails.

3.4.4. Vigour and fecundity

The analysis of the plant characteristics in both sites revealed that there was no one group of plants showing significantly greater vigour or fecundity than the others. There was also variation in vigour and fecundity between the Cataract and Darkes Forest populations, confirming that comparison of these two sites is difficult. The results certainly reflect the obvious differences in the sites: the drier, relatively higher Darkes Forest site, shows that the *B. oblongifolia* and the hybrid plants are larger and more fertile than the *B. robur* plants, while *B. robur* plants are generally larger in the wetter Cataract site, but are still not as fecund as the hybrids or *B. oblongifolia* plants.

In terms of magnitude in the Darkes Forest site, *B. oblongifolia* and the hybrid origin plants are consistently larger and more fecund, in the analyses using both indices to categorize the plants. The plants of hybrid origin are consistently higher, potentially giving them the advantage in competition for light. At the Darkes Forest site, the hybrids also have the greatest maternal fecundity, as the hybrid plants show the greatest number of seeds per cone. *B. oblongifolia* plants appear to have the greatest paternal fertility, or pollinating potential, as they have the largest number of inflorescences per plant. In the

total plant array (where the plants are categorized using the morphological index), the hybrids generally exhibit the most variability (as can be seen by the standard error of the means Tables 3.10 and 3.11) in three characters measured.

The results from the Cataract site show greater variability in the vigour and fecundity, both within and between the groups, than the vigour and fecundity of the plants within the Darkes Forest site. Under both indices, *B. oblongifolia* exhibits the greatest potential for maternal fertility, while the hybrid plants have the greatest paternal potential. Generally, the hybrids show the greatest mean plant height, but are not significantly taller than the other morphs in any analysis.

3.4.5. Spatial variation in phenotype

According to the environmental cline model, two "species" or "genetic types" occupy and are fitter at two extremes of the cline - along the cline, one species is gradually replaced by the other. Under these circumstances, it is expected that each species will show greater vigour and fecundity within its own territory, which will decrease along the cline as the territory of the other species (whose vigour and fecundity is increasing) is approached. Similarly, the vigour and fecundity of the "hybrid" will peak at the centre of the cline and subsequently drop away. This pattern was not found within the *B. robur/B. oblongifolia* complex. There was some evidence on the edges of the clines that there was an increase in the mean of the characters measured for the parental type plants, but this probably reflected the lower numbers of the other types of plants within the quadrat. While the hybrid plants never became more abundant than the parental plants in the centre of the cline (indicating that they may be competitively inferior to the parental plants), they did not show reduced vigour or fecundity. Several hypotheses may explain this apparent equivalent vigour and fecundity between plant classes: (i) The scale at which the populations were observed may not have been large enough to detect differences between the plant types. Ideally, characters within the pure stands need to be observed, but the comparison of pure stand and hybrid zone in this study would not be valid, as the

populations have experienced differences in fire regime, making comparisons impossible. (ii) There may really be no difference in the fitness between the parents and the hybrid plants. In the total populations, analysed above, the hybrids are often taller and show higher levels of fertility, but these figures are not significantly different to the other plant types. (iii) The hybrid zone may be environmentally heterogeneous on a fine scale, so that the area as a whole may be able to support the growth of all three plant types, which are suited to different micro-habitats, within a seemingly limited area. This may be linked to the second hypothesis, in that the hybrids may have some advantage in these areas (supported by the fact that the hybrids had the highest mean in most of the measured characters), but the ecotone is well within the range of tolerance of the parental species (Endler 1977).

3.4.6. Dispersal and Selection

The rates of dispersal estimated within the *B. robur/B. oblongifolia* clines were about five times larger than the width of the cline in each case. Wright (1943, 1946) considered that large dispersal or gene flow distances between groups prevented character differentiation that might arise through isolation, or negated any differentiation that had already occurred. Endler (1973, 1977) has suggested that differentiation is possible under extensive gene flow, and therefore a narrow cline formed between species can be maintained if one species is better adapted to one environment, and the other species to another.

There is the assumption in this study that the recombination rate is 0.5. This assumption seems to be fairly well grounded because, although there is linkage between loci in the centre of the cline, the loci seem to be in linkage equilibrium in the pure stands, away from the cline.

A striking result of this study is that the estimates for dispersal distance and cline width are very different between sites, perhaps indicating that there are site specific

characteristics, which are fundamental to the breeding system and fitness of the plants (such as the environment, pollinator type or activity). The estimates of dispersal obtained using the cline shape will be used as prediction for gene flow determined in Chapter Five, through pollinator foraging behaviour and marker allele dispersal within the two populations.

High levels of selection of hybrid progeny within the hybrid zone was suggested in many aspects of this study. Firstly, the significant deficit of heterozygotes within the hybrid zones, reinforced by the result of no significant deviation from Hardy-Weinberg equilibrium in the pure *B. oblongifolia* populations, suggests that there is strong hybrid heterozygote disadvantage within the hybrid zones. This is reinforced by the high linkage disequilibrium detected within the cline (and the linkage equilibrium outside the cline) and the coefficient of effective selection (s*) on the loci within the hybrid zone.

The evidence collected so far to support that the hybrid zone formed by *Banksia robur* and *Banksia oblongifolia* may be the result of the balance between selection against hybrids and dispersal into the hybrid zone, or environmental cline is presented in Table 3.12. The results presented in this Chapter do not support one model over the other, indicating that this hybrid zone is the result of complex interaction between the environment and hybrid disadvantage The data presented in this Chapter, however, represent the genotypic variation and fitness and fecundity measured from plants already established in the population. Selection may also act before the establishment of the plant: at the pre-pollination, pre-zygotic and the germination phases of the life-cycle. These phases within *Banksia robur*, *Banksia oblongifolia* and their hybrid zone will be investigated in the next section of the thesis.

Table 3.12. Evidence collected for the *B.robur/B. oblongifolia* clines so far supporting the two models put forward explaining hybrid zone formation and maintenance and the corresponding source of the information.

Environmental Cline	**Tension Zone**
*Formation of mosaic hybrid zone	*Coincidence of locus clines
*No differences in fitness between the parentals and the hybrids on the scale of the cline	*Deficit of hybrid heterozygotes
*Narrowness of cline	*Linkage disequilibrium in centre of cline
	*High selective pressure in centre of cline

Chapter Four

Flowering and fruiting phenology within the *Banksia robur/Banksia oblongifolia* hybrid zone

4.1. Introduction

The reinforcement of pre-zygotic reproductive isolation is the predicted outcome of selection against hybrids within a hybrid zone (Littlejohn 1981), at which stage the speciation will be complete. The previous Chapter suggested that there may have been some selective force acting against the hybrids formed by *Banksia robur* and *B. oblongifolia*. Detailed study of certain aspects of the life-cycle, such as flowering time (in this Chapter), forager constancy to a single species (next Chapter) and pollen incompatibilities (Chapter Six), may determine if and when selection is occurring, and the degree of isolation of these species.

4.1.1. Reproductive isolation

The study of reproductive isolation in hybridizing species is common within animals (Littlejohn *et al.* 1971, McDonnell *et al.* 1978, Harrison 1985, Shaw *et al.* 1986, Baker & Baker 1990, Bendix & Howard 1991). This isolation can come in the form of differences in mating rituals, temporal reproductive non-synchrony and postmating genetic incompatibilities. In sympatric species of plants, the development of asynchronous flowering is the primary form of isolation, particularly in communities of species where pollinators are inconstant pollen or nectar feeders. However, most studies on flowering phenology of sympatric species have been concerned with the change in flowering times as a result of competition for pollinators (Mosquin 1971, Ågren & Fagerström 1980). Some studies suggest that flowering will become more distinct within sympatric species and will be staggered throughout the year as a response to this competition (Mosquin 1971, Stiles 1977, Feinsinger 1978, Whelan & Burbidge 1980, Copland & Whelan

1989), while others hypothesise that, in a mutualistic response, increased flowering synchrony will increase the attraction of the community to pollinators as a whole (Rathcke 1983, Gross 1990). However, flowering times for pairs of species that are sympatric and hybridizing species has not been addressed as widely as is probably warranted (but see Hopper and Burbidge 1978; Drake 1980)

4.1.2. Reinforcement

Historically, speciation of two populations was thought to be enhanced through reinforcement of reproductive isolation, as a result of the selection against hybrids (Dobzhansky 1951). Currently, however, speciation through reinforcement has little support (Paterson 1978, 1982, Butlin 1989, Sanderson 1989), primarily because there are no convincing examples of reinforcement within natural populations. However, as Harrison & Rand (1989) point out, the lack of evidence may not necessarily indicate that it is an unlikely outcome, but just that reinforcement is difficult to demonstrate or that it may occur in restricted conditions. Indeed, Butlin (1989) suggests that the conditions available within mosaic hybrid zones, such as the *Banksia robur/B. oblongifolia* complex, may increase the probability of reinforcement.

Butlin (1989) suggests that for a system to convincingly demonstrate reinforcement, there must be: present or past gene flow between species and divergence of mate recognition system components where the species are in contact, and since the time of contact. This mate recognition system divergence must be large enough to decrease production of unfit hybrids, and should not be the result of any other selection pressure. In the *Banksia* system under study, there has been gene flow between the species in the past, as evidenced by the presence of hybrids within the hybrid zones detected in Chapter Two. In this Chapter, flowering time is the component of the mate recognition system that will be observed, while the current potential for hybridization will be assessed from the seed set within the observed plants. The other important potential component of the mate

recognition system used by flowering plants, the attraction of specific pollen vectors, will be addressed in the following Chapter.

To determine if there is any evidence of reinforcement between species, and to assess intra- and inter-population variation in flowering and seed set, a comparison of flowering times of the parental species in the hybrid zone and allopatric populations is necessary. Divergence between the species in flowering time only in areas where the two species overlap may suggest that separation of species may be occurring as a result of selection against hybrid progeny) (Caisse and Antonovics 1978). Alternatively, there may be the tendency for the character to become more similar when in the same environment (Endler 1982).This comparison of species within a hybrid zone and the same species in a pure stand is sometimes used when comparing morphological traits for both plants and animals (e.g. Drake 1980; Doyle and Doyle 1988), but has been used rarely to test for reproductive isolation traits.

4.1.3. Flowering time as a control of the amount of hybridization

Within a plant hybrid zone, overlap of flowering times may be the primary determinant of the amount of introgression, because even if pollinators forage in both species and there are no barriers to interspecific matings, flowering synchrony is initially necessary for interspecific pollination to occur.

Determinations of flowering phenologies are generally made at the level of the population (Fox 1989). This approach is not sufficiently detailed for studies of hybridization, where it is also desirable to estimate the synchrony of flowering of individual plants and in some instances individual flowers or inflorescences, because only at this level can the true amount of interspecific gene exchange and introgression be estimated. Furthermore, even if there is overlap in flowering between two species in a hybrid zone, gene exchange will depend upon successful fruit set. Hence assessment of flowering time of those flowers or

inflorescences which do produce seeds will provide greater precision in the estimation of potential introgression in the population.

4.1.4. Chapter aims

This Chapter documents an hierarchical assessment of the overlap of flowering phenologies in *Banksia robur* and *B. oblongifolia* and their hybrids, and combined these with details of seed set in order to estimate the potential for hybridization in this system. Specifically, in each of two flowering seasons, differences in flowering time of the parental morphs found within two hybrid zones and within adjacent monospecific stands have been assessed. Further, the degree of synchrony of flowering among individual inflorescences within the hybrid zones has been examined and the time of flowering of those inflorescences that set fruit was determined. From this examination, an assessment of the possibility for gene flow between the two species, what effect sympatry has on the flowering phenology of the two species, the possible extent of gene flow between the species and the hybrids, and the possible proportions of hybrids and backcrossed individuals within the seeds produced within the season.

4.2. Methods

4.2.1. Plant selection

The study was conducted in the pure stands and hybrid zones of Darkes Forest and the Cataract Catchment, described in Chapter One.

Flowering of a sample of individuals within the two mixed stands of *B. robur, B. oblongifolia* and hybrids was monitored. Similar numbers of plants within each grouping were randomly chosen from the plants already tagged in each hybrid zone. The final number of plants within each group monitored over the two flowering seasons is listed in Table 4.1.

Table 4.1. The number of plants observed within the hybrid zones and pure stands, at Darkes Forest and within the Cataract catchment. All the plants had been identified genetically and given a score on the genetic hybrid index: those within the *B. robur* group all had GHISs of 0, hybrids had GHISs of 1, 2, 3 and 4, and those within the *B. oblongifolia* group had GHISs of 5 and 6. The same plants were monitored over the two flowering seasons in 1989-90 and 1990-91.

		Number of plants	
		Cataract	Darkes Forest
Hybrid zone	***B. robur***	22	21
	Hybrid	20	23
	B. oblongifolia	16	17
Pure stand	***B. robur***	23	23
	B. oblongifolia	23	23

Each plant had been genetically identified, and allocated a genetic hybrid index score (GHIS), according to the methods in Chapter Two. The plants were then grouped as either *B. robur* (GHIS=0), *B. oblongifolia* (GHIS=5 or 6) or hybrid (GHIS=1 to 4 inclusive), and these groupings are used in this Chapter.

4.2.2. Monitoring of flowering

4.2.2.1. Data collection

To compare the flowering times of the parental species in the hybrid zone and the pure stands, more plants of each parental species were randomly selected from 100 individually identified plants, in stands of each species that were at least 100 metres away from the hybrid zones.

Each plant was monitored weekly, and the stage of opening of each of its inflorescences was recorded over two flowering seasons from December 1989 to June 1990 and from December 1990 to June 1991. For ease of comparison between years and populations, week 1 was assigned to the second week in December, which was the earliest time any inflorescence was open. An inflorescence was considered to be "flowering" when at least a few flowers were open and the inflorescence could be either pollen a donor or receiver.

Generally, each inflorescence observed was found to have completed flowering within two weeks (only two inflorescences from all observed in two seasons took greater than one week to complete flowering), so only one observation of "opened" was recorded for each inflorescence. The flowering consistency of plants within the sample was assessed by determining the number of plants to have flowered in both, either or neither of the years in which flowering was observed.

Each week, the number of "open" inflorescences was tallied for each species, and the mode and median of the flowering time of each morph, in each population, in each year, was obtained from the distribution of the number of inflorescences per week. The mean is

the traditional and most commonly used measure to describe the central tendency of a set of data (Zar 1984). However, as the distribution of these data was skewed, it was thought that the more appropriate measure of the central tendency of this set of data was the median (Zar 1984).

4.2.2.2. Statistical analysis

Heterogeneity in flowering patterns between morphs, stands, years and populations was assessed using Model I Three Factor Analyses of Variance (as all factors were fixed). Differences in the time of flowering among morphs was determined using the week of flowering of each inflorescence within the ANOVA. Flowering time at the level of the inflorescence was used rather than at the level of the plant, as it was assumed that the time of flowering of each inflorescence was independent of other the flowering time of other (past, present and future) inflorescences on the same plant. Each inflorescence was also considered to be the unit of pollen reception. A Tukey test was employed to determine which factors contributed to any significant differences detected (Zar 1984). Heterogeneity in the flowering times of the pure stand and hybrid zone of each parental species was tested in separate analyses.

4.2.2.3. Assessment of flowering synchrony

The flowering pattern of each hybrid zone population was assessed using the number of inflorescences flowering per week for each morph in each population (Table 4.2). The flowering overlap of each individual inflorescence (within its week of flowering) was assessed by counting the numbers of inflorescences of each morph also in flower at that time. The mean number of other inflorescences open simultaneously with each inflorescence was then taken within the morph, over the whole season. To relate this overlap to the relative abundance of the morph in each hybrid zone population, the mean number of other inflorescences open was then multiplied by the proportion of the number of plants within each morph to the total within each hybrid zone.

Table 4.2. Method for determining the synchrony of flowering of each morph. **A.** The raw data used in this example. The first column is a hypothetical list of the individual inflorescences (inflorescence R1, R2, etc.) observed to be opened in a particular week (R= *B. robur*, H=hybrid, O= *B. oblongifolia*). The other columns show the number of other *B. robur*, hybrid and *B. oblongifolia* inflorescences open at the same time as R1, etc. **B.** These numbers were then used to determine a figure for the mean number of inflorescences open synchronously for each morph. This mean was then multiplied by the proportion of plants within each morph observed to the total within the hybrid zone.

A.

	R	**H**	**O**
R1	5	2	3
R2	7	3	1
R3	4	2	4
H1	2	3	5
H2	3	4	5
O1	2	2	7
O2	3	3	6
O3	1	2	6

B.

	Inflorescence		
	B. robur	**Hybrid**	***B. oblongifolia***
B.robur	Mean 5,7,4	Mean 2,3,2	Mean 3,1,4
Hybrid	Mean 2,3	Mean 3,4	Mean 5,5
B. oblongifolia	Mean 2,3,1	Mean 2,3,2	Mean 7,6,6

4.2.3. Potential for hybridization and introgression

Fruit set was recorded when follicles were fully developed (between 6 and 12 months after commencement of flowering). The number of infructescences per plant and the number of follicles per infructescence were used as measures of the relative reproductive success of the parents and the hybrids.

The proportion of parental, hybrid and backcrossed progeny produced in each cohort can be indirectly predicted, using the flowering time and fruit set data. These predictions are based on the assumption that the pollen from all three types of plants within each hybrid zone have equal chance of germinating on the stigma upon which it is deposited. The probability that the observed fruit set resulted from pollination (i) within the species, (ii) between the species and (iii) between the species and the hybrid was estimated by comparing the proportion of inflorescences from that and the other morphs flowering simultaneously. For example, for *B. robur* infructescence 1, the number of other *B. robur* inflorescences, hybrid inflorescences and *B. oblongifolia* inflorescences in flower in the same week were assumed to represent the proportion of each pollen type available in the pollen pool. These proportions were used to estimate the number of fruits produced by *B. robur* infructescence 1 as a result of pollination by each morph. This procedure was used for each infructescence produced by each morph, each year, in each population. The cumulative estimate for each infructescence provided an estimate of the potential proportion of fruits which could be produced as a result of interspecific pollination and introgression.

4.3. Results

4.3.1. Flowering of individual inflorescences

4.3.1.1. Variation in peak flowering times

The distributions of flowering times differed markedly and consistently for populations of *Banksia robur* and *B. oblongifolia* (Figure 4.1). *Banksia robur* was in both sites in both years the first to peak, between Weeks 4 and 8. *B. oblongifolia* peaked between Weeks

Figure 4.1. Flowering times of the *Banksia robur, B. oblongifolia* and the hybrid over two flowering seasons: a. the Cataract hybrid zone parental species; b. Cataract hybrid zone hybrid plants c. Cataract pure stand parental species; d. Darkes Forest hybrid zone parental species; e. Darkes Forest hybrid zone hybrid plants; f. Darkes Forest pure stand parental species. The 1989-90 season is represented by the distribution from week 0, and the 1990-91 season is represented by the distribution from week 53.

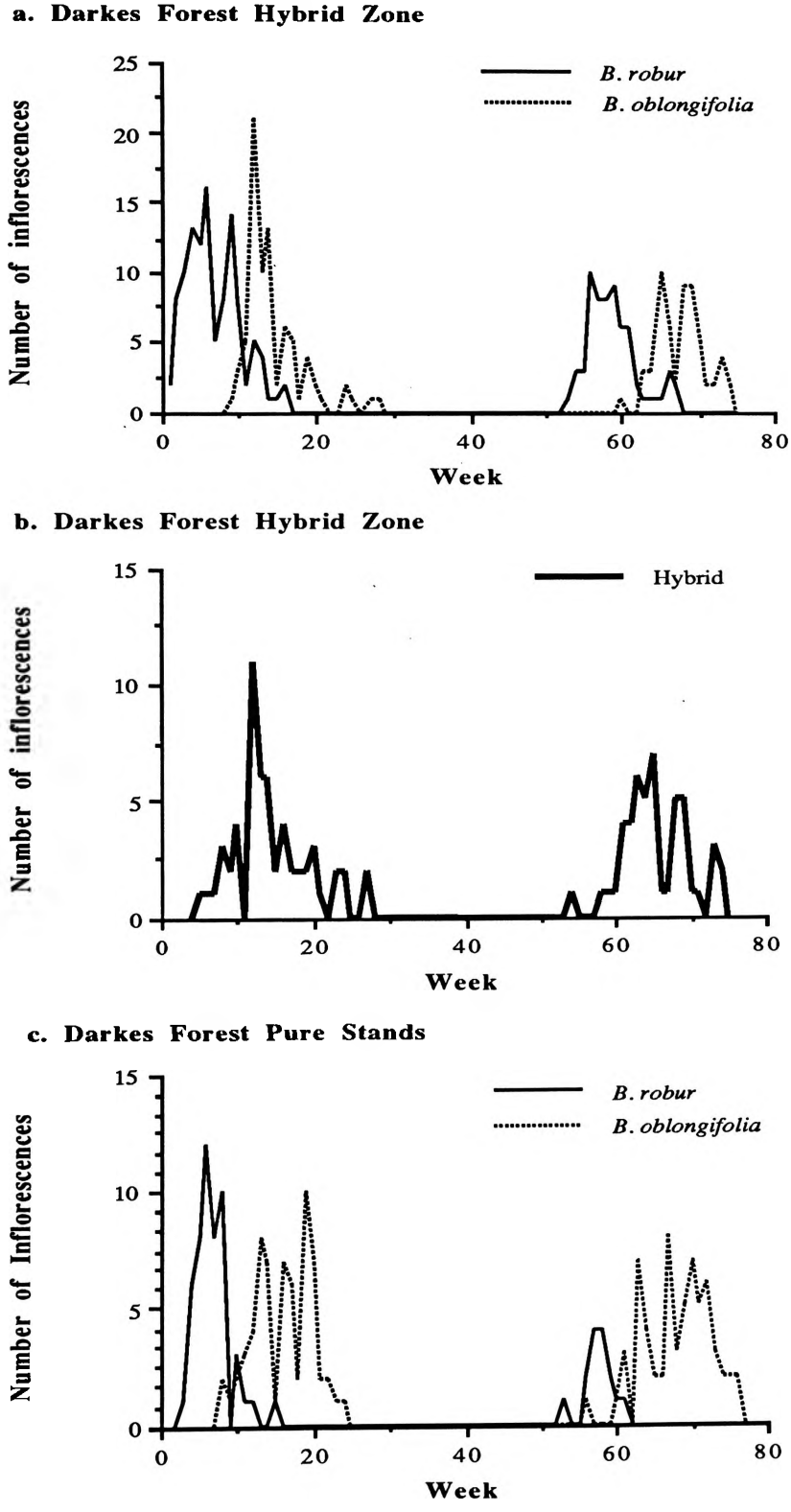
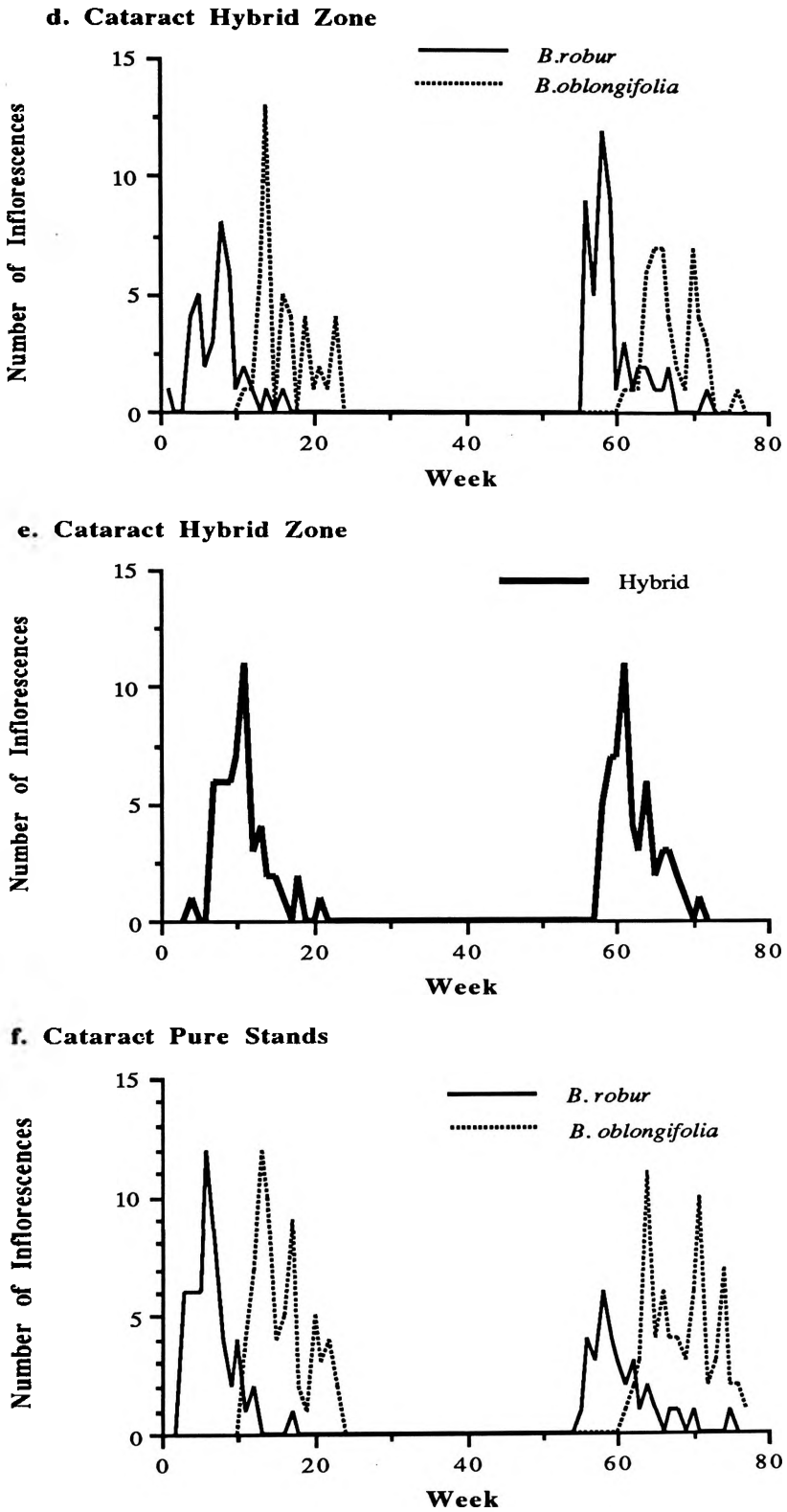


Figure 4.1. continued



12 and 17. The peak flowering time of the hybrid populations lay between the peaks of the two species within the hybrid zone, except in the hybrid zone population at Darkes Forest in both seasons, where the hybrid peak corresponded with the peak of *B. oblongifolia.*

The clear difference between the flowering patterns of the hybrids and the parental species were reflected by differences in the median flowering times (Table 4.3). In the hybrid zones, the difference in median flowering time between the two species was between 6 and 9 weeks. The median of the hybrid flowering distributions suggest that the flowering time of the hybrid is not exactly intermediate between the parental species. The medians of hybrid flowering were between 2 and 7 weeks after the median for *B. robur* and between 0 and 5 weeks before the median for *B. oblongifolia.* However, the median flowering times of the hybrids were not consistently closer to the median flowering time of either parent species.

There was great similarity in flowering time distributions of the parental species within the hybrid zones and those within the pure stands (Figures 4.1c and 4.1f). The mode and median of these distributions confirm the similarities detected in the graphical representations (Table 4.3). There is a maximum difference of 2 weeks between the modes and median in the hybrid zones and pure stands of *B. robur.* There was more variation in the differences between hybrid zone and pure stand *B. oblongifolia,* with the maximum difference between the mode and median are 7 and 3 weeks, respectively. Duration of flowering time was inconsistent between species, stands and populations (Table 4.3).

4.3.1.2. Timing of flowering

The flowering time of the individual inflorescences reflected the differences between morphs obtained for the whole population, indicating that the differences were not just a result of the mean effect over the whole population, but were consistent between

Table 4.3. The mode and median week of flowering, and the range and duration of flowering for *Banksia robur*, *B. oblongifolia* and the hybrid in the hybrid zones, and the two species in the pure stands. The range of flowering time is the number of weeks between the flowering of the first and last inflorescences (which are shown in the brackets under the range) inclusive. The figures are for the two populations observed, over the two flowering seasons studied. (See Table 4.1 for sample sizes).

	Pure Stand	Hybrid Zone			Pure Stand
	B. robur	*B. robur*	Hybrid	*B. oblong.*	*B. oblong.*
			Cataract		
1989-1990					
Mode	6	8	11	14	13
Median	6	8	10.5	15	15
Range	15 (3-17)	13 (4-16)	12 (4-15)	13 (11-23)	13 (11-23)
1990-1991					
Mode	6	6	9	13, 14, 18	12
Median	7	6.5	9	14	17
Range	21 (3-23)	17 (4-20)	13 (6-18)	16 (9-24)	19 (9-27)
			Darkes Forest		
1989-1990					
Mode	6	6	12	12	19
Median	6.5	6	13	13	16
Range	13 (3-15)	17 (1-17)	23 (5-27)	20 (9-28)	17 (8-24)
1990-1991					
Mode	5, 6, 7	4	13	13	16
Median	6	6	13	16	18
Range	10 (1-10)	21 (1-21)	21 (2-22)	15 (8-22)	22 (4-25)

individual plants. The effects of population, flowering season and morph on the time of flowering, assessed using a 3-factor ANOVA (Table 4.4), revealed significant differences between populations, morphs, and in the interactions morph x year and morph x flowering season x population. A Tukey test revealed that differences between all combinations of pairs of morphs were significant. The significant differences detected in the interaction of morph x population were due to the difference in the mean week of flowering of the hybrids.

The effect of population, season and stand of each parental morph was assessed using a 3-factor ANOVA (Table 4.5). For *Banksia robur*, no significant difference was detected between the flowering time of the pure stand and hybrid zone inflorescences (Table 4.5a). There were significant differences between the populations, and between the stands in each flowering season, each year. However, there was a significant difference detected between the hybrid zone and pure stand *B. oblongifolia* (Table 4.5b).

4.3.2. Consistency of flowering of plants between years

The consistency of flowering of individual plants between flowering seasons may indicate fluctuations in the level of flowering each year, which has been shown in previous studies to alter significantly between seasons (Copland & Whelan 1989). A large proportion of plants within the hybrid zones have the ability to flower in consecutive years, as a majority of the plants observed (about 65%) flowered in both seasons surveyed (Figure 4.2). Only 11.8% of the total number of plants observed flowered in neither season.

4.3.3. Synchrony of flowering within and between morphs

For each open inflorescence of each parental species, the majority of other inflorescences opened in the hybrid zone at the same time were of the same species (Table 4.6). For every *B. robur* inflorescence open, there was (depending on the population and year) a

Table 4.4. Three-factor ANOVA results for week of flowering of each inflorescence. The factors were population (Cataract and Darkes Forest), year or flowering season studied, and morphs (*B. robur*, hybrid and *B. oblongifolia*). The degrees of freedom (D.F.), mean square (M.S.) and F value are shown for each source of variation. Levels of significance: NS: not significant; *= p≤0.05; **= p≤0.01; ***= p≤0.001.

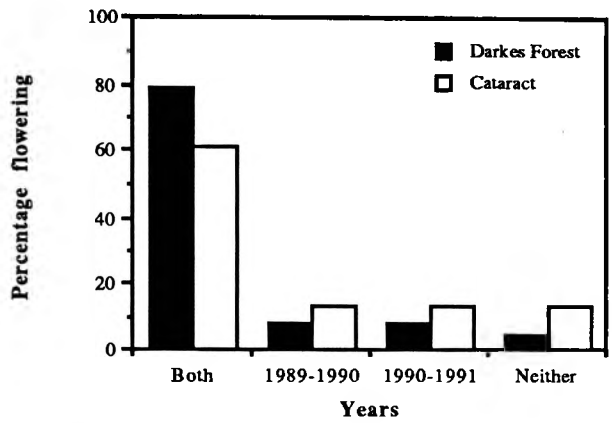
Source of Variation	D.F.	M.S.	F	P
Population	1	108.681	8.353	**
Year	1	14.51	1.115	NS
Population X Year	1	1.315	0.101	***
Morph	2	3889.811	298.973	***
Morph X Population	2	251.33	19.317	NS
Morph X Year	2	7.645	0.588	*
Morph X Population X Year	2	51.916	3.99	*
Error	697	13.011		

Table 4.5. Three-factor ANOVA results for the week of flowering of each inflorescence of a. pure stand and hybrid zone *B. robur* and b. pure stand and hybrid zone *B. oblongifolia*. Levels of significance: NS: not significant; *= p≤0.05; **= p≤0.01; ***= p≤0.001.

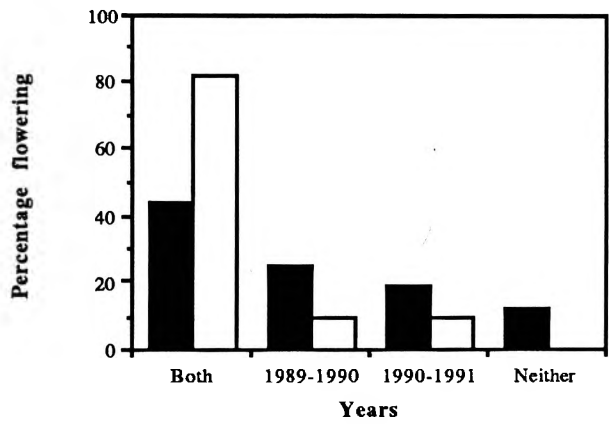
Source of Variation	D.F.	M.S.	F	P
a. *B. robur*				
Population	1	58.865	5.272	*
Year	1	4.318	0.387	NS
Population X Year	1	24.75	2.216	NS
Morph	1	20.539	1.839	NS
Morph X Population	1	5.716	0.512	NS
Morph X Year	1	21.12	1.891	NS
Morph X Population X Year	1	55.552	4.975	*
Error	430	11.166		
b. *B. oblongifolia*				
Population	1	2.705	0.192	NS
Year	1	43.244	3.073	NS
Population X Year	1	25.888	1.89	NS
Morph	1	133.518	9.487	**
Morph X Population	1	15.668	1.113	NS
Morph X Year	1	32.659	2.321	NS
Morph X Population X Year	1	45.331	3.221	NS
Error	430	14.074		

Figure 4.2. The consistency of flowering of individual plants between years. The histograms show the number of plants that flowered in both the 1989-90 and 1990-91 flowering seasons, only one of the two seasons, or neither season. The histograms are a. for *B. robur* plants, b. the hybrid plants and c. the *B. oblongifolia* plants within the hybrid zones in the two populations surveyed.

a. B. robur



b. Hybrid



c. B. oblongifolia

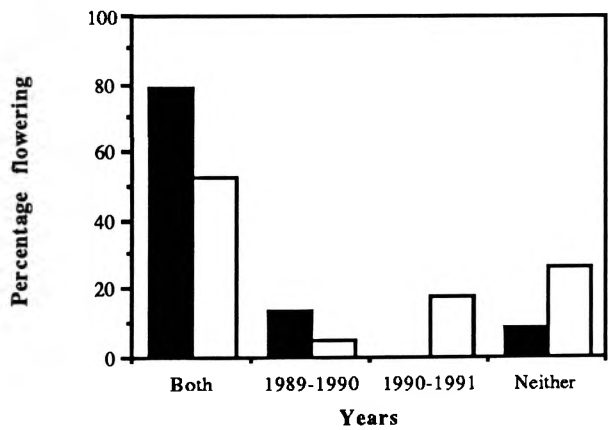


Table 4.6. An estimate of the overlap of flowering time of each morph within each hybrid zone, calculated for each population, in each flowering season (1989-90 and 1990-1991), based on the example presented in Table 4.2. The column headers represent the reference inflorescences, while the rows are the mean number of other inflorescences within each category open simultaneously with each inflorescence, which were then multiplied by a correction factor which was the proportion of each morph observed to the total number of the morph within the population. The correction factors for each morph in each population were: Cataract - *B. robur*, 0.35; Hybrid, 0.38; *B. oblongifolia*, 0.73; and Darkes Forest: *B. robur*; 0.55; Hybrid, 0.62; *B. oblongifolia*, 0.68.

	1989-1990			1990-1991		
	B. robur	**Hybrid**	***B.oblongifolia***	***B. robur***	**Hybrid**	***B.oblongifolia***
Cataract						
B.robur	1.4	1.5	0.3	2.9	1.2	0.3
Hybrid	1.6	1.8	0.4	1.7	1.9	0.9
B.oblongifolia	0.37	0.7	4.8	0.7	1.5	3.9
Darkes Forest						
B.robur	5.0	0.9	1.4	3.1	0.6	1
Hybrid	2.7	1.6	5.3	1.4	1.2	4.8
B.oblongifolia	2.4	2.8	8.2	0.68	1.6	7

mean of between 1.4 and 5.0 other *B. robur* inflorescences flowering simultaneously, but only between 0.3 and and 1.4 *B. oblongifolia* inflorescences. In contrast, for every *B. oblongifolia* inflorescence open there was a mean of between 4.8 and 8.2 other *B. oblongifolia* inflorescences and between 0.5 to 3.5 *B. robur* inflorescences open simultaneously.

The mean number of hybrid inflorescences flowering at the same time as inflorescences of either parent species varied between populations. In the Cataract population, more hybrid inflorescences were open simultaneously per *B. robur* inflorescence than per *B. oblongifolia* inflorescence. However, there was a higher mean number of hybrid inflorescences open at the same time as each *B. oblongifolia* inflorescence than each *B. robur* inflorescence in the Darkes Forest population (Table 4.6).

Differences were observed between populations in the number of parental morph inflorescences open per flowering hybrid inflorescence. There was a larger mean number of *B. robur* inflorescences open than *B. oblongifolia* inflorescences per open hybrid inflorescence, in the Cataract population than in the Darkes Forest population. The mean number of hybrid inflorescences open per open hybrid inflorescence was higher in the Cataract population (1.8 and 1.9) than in the Darkes Forest population (1.6 and 1.2).

4.3.4. Fruit set

There were significant differences between the morphs in the number of inflorescences produced per plant (Table 4.7). There were fewer *B. robur* inflorescences per plant than the other morphs (Table 4.7a).

Generally, there was great variation in the proportion of inflorescences that set fruit (Table 4.8). Within the hybrid zone, the percentage of inflorescences that became infructescences varied between 18.8% and 47.8% in *B. robur*, 16.3% and 27.3% among the individuals of hybrids and 7.3% and 34.0% in *B. oblongifolia*. The most surprising

Table 4.7. Three-way ANOVA results for the number of a. inflorescences, b. infructescences and c.follicles. The factors were population (Cataract and Darkes Forest), year or flowering season studied, and morphs (*B. robur*, hybrid and *B. oblongifolia*). The degrees of freedom (D.F.), mean square (M.S.) and F value are shown for each source of variation. Levels of significance: NS: not significant; *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

Source of Variation	D.F.	M.S.	F	P
a. Inflorescences				
Population	1	4.309	0.501	NS
Year	1	22.744	2.642	NS
Population X Year	1	33.131	3.849	NS
Morph	4	33.03	3.837	**
Morph X Population	4	32.93	3.826	**
Morph X Year	4	3.663	0.426	NS
Morph X Population X Year	4	4.93	0.557	NS
Error	402	8.608		
b. Infructescences				
Population	1	1.973	2.374	NS
Year	1	0.385	0.463	NS
Population X Year	1	2.033	2.447	NS
Morph	4	4.955	5.963	***
Morph X Population	4	2.241	2.696	*
Morph X Year	4	0.282	0.339	NS
Morph X Population X Year	4	0.044	0.053	NS
Error	402	0.831		
c. Fruits				
Population	1	27.601	0.022	NS
Year	1	647.73	0.519	NS
Population X Year	1	4331.192	3.472	NS
Morph	4	5248.343	4.207	**
Morph X Population	4	4933.248	3.954	**
Morph X Year	4	447.747	0.359	NS
Morph X Population X Year	4	195.958	0.157	NS
Error	402	1247.536		

Table 4.8. The relative reproductive success of the *B. robur*, *B. oblongifolia* and the hybrid in the hybrid zone, and the two species in the pure stands in a. the Darkes Forest site and b. the Cataract catchment site. The table shows the total number of inflorescences produced by the plants observed within each site, mean number of inflorescences (± standard error) per plant surveyed within the site, total number of infructescences produced by the surveyed plants, mean number of infructescences (± standard error) produced per surveyed plant, the percent of inflorescences that produced seed, the total number of follicles produced by the surveyed plants and the mean number of follicles (± standard error) per surveyed plant (the original plant sample sizes are given in Table 4.1).

	Pure Stand *B.robur*	*B.robur*	Hybrid Zone Hybrid	*B.oblongifolia*	Pure Stand *B.oblongifolia*
a. Darkes Forest					
1989-1990					
Inflorescences/site	55	111	57	80	68
Mean inflors/plant	2.4(±0.4)	5.3(±1.2)	2.5(±0.5)	4.7(±0.7)	3.0(±0.8)
Infructescences/site	9	19	9	5	13
Mean infructs/plant	0.4(±0.1)	0.9(±0.3)	0.4(±0.1)	0.3(±0.1)	0.6(±0.1)
% conversion	16.4	17.1	15.8	6.3	19.1
Follicles/site	259	923	286	78	585
Mean follicles/infruct.	28.8(±3.8)	48.6(±6.1)	31.8(±3.4)	15.6(±2.3)	45.0(±7.4)
1990-1991					
Inflorescences/site	18	64	49	58	62
Mean inflors/plant	0.8(±0.3)	3.1(±0.5)	2.1(±0.6)	3.4(±0.9)	2.7(±0.5)
Infructescences/site	0	13	10	3	8
Mean infructs/plant	-	0.6(±0.2)	0.4(±0.1)	0.2(±0.1)	0.4(±0.1)
% conversion	0	20.3	20.4	5.2	12.9
Follicies/site	-	515	287	50	322
Mean follicles/infruct.	-	39.6(±5.4)	28.7(±4.0)	16.7(±4.0)	40.3(±10.9)

Table 4.8. continued

b. Cataract

1989-1990		
Inflorescences/site	49	35
Mean inflors/plant	2.1(±0.4)	1.5(±0.3)
Infructescences/site	7	17
Mean infructs/plant	0.3(±0.1)	0.8(±0.2)
% conversion	14.3	48.6
Follicles/site	219	396
Mean follicles/infruct.	31.3(±9.3)	23.3(±3.4)
1990-1991		
Inflorescences/site	32	55
Mean inflors/plant	1.4(±0.3)	2.5(±0.5)
Infructescences/site	8	17
Mean infructs/plant	0.4(±0.1)	0.8(±0.2)
% conversion	25	30.9
Follicles/site	232	495
Mean follicles/infruct.	29(±5.9)	29.1(±2.7)

56	42	69
3.5(±0.6)	2.1(±0.8)	3.0(±0.6)
15	11	2
0.9(±0.3)	0.6(±0.3)	0.1(±0.1)
26.8	26.2	2.9
330	447	54
22(±2.8)	40.6(±3.3)	27.0(±17.0)
55	46	74
3.4(±0.8)	2.3(±0.8)	3.2(±0.7)
13	17	3
0.8(±0.4)	0.9(±0.4)	0.1(±0.1)
23.6	37.0	4.1
369	530	123
28.4(±3.2)	28.2(±2.9)	41(±18.1)

result was the difference in infructescence production between the species in the pure stands and hybrid stands of the parental species. In both *B. robur* and *B. oblongifolia,* the individual plants observed in the pure stands had lower infructescence production than those in the hybrid zone: the percentage of inflorescences that became infructescences in the pure stand *B. robur* individuals were between 0% and 25.2%, and between 2.7% and 19.3% in the pure stand *B. oblongifolia* individuals. Furthermore, the number of infructescences per plant varied significantly among morphs (Table 4.7b). A Tukey test revealed that the number of infructescences per plant of both *B. robur* and *B. oblongifolia* pure stands were significantly different to only the hybrid zone *B. robur*, which produced the greatest number of infructescences per plant.

There were significant differences in the number of follicles produced per plant among the morphs ($p<0.01$), and among the morphs in each population ($p<0.01$) (Table 4.7c). Again it was the pure stand *B. robur* and *B. oblongifolia* that were significantly different to the hybrid zone *B. robur*.

There seemed to be well-defined time spans in which the inflorescences of each parental species that eventually set fruit were in flower (Figure 4.3). In each population, in each flowering season, *B. robur* infructescences had generally flowered no later than week 11.

There were a number of infructescences of each parental species that flowered during the flowering time overlap. The largest overlap was apparent in the Darkes Forest population in the 1990-1991 flowering season (Figure 4.3d), where 26.7% of the *B. robur* inflorescences that set fruit flowered while *B. oblongifolia* was in flower. In other distributions, 15.8% (Figure 4.3a), 16.7% (Figure 4.3b) and 26.1% (Figure 4.3c) of *B. robur* infructescences flowered within the flowering time of *B. oblongifolia.*

The percentages of *B. oblongifolia* infructescences flowering while *B. robur* was in flower were larger than the percentages of *B. robur* infructescences flowering while *B.*

Figure 4.3. The week of flowering of each infructescence: a. the Cataract population in 1989-90 season; b. Cataract population in 1990-91 season; c. the Darkes Forest 1989-90 season; d. the Darkes Forest 1990-90. The black bars represent *Banksia robur* inflorescences, the stippled bars are the hybrid inflorescences and the white bars are *Banksia oblongifolia* inflorescences. The vertical broken lines are the boundaries of the overlap in flowering time - the first line is the first week *Banksia oblongifolia* was in flower, and the second line is the last week *B. robur* was in flower for each distribution.

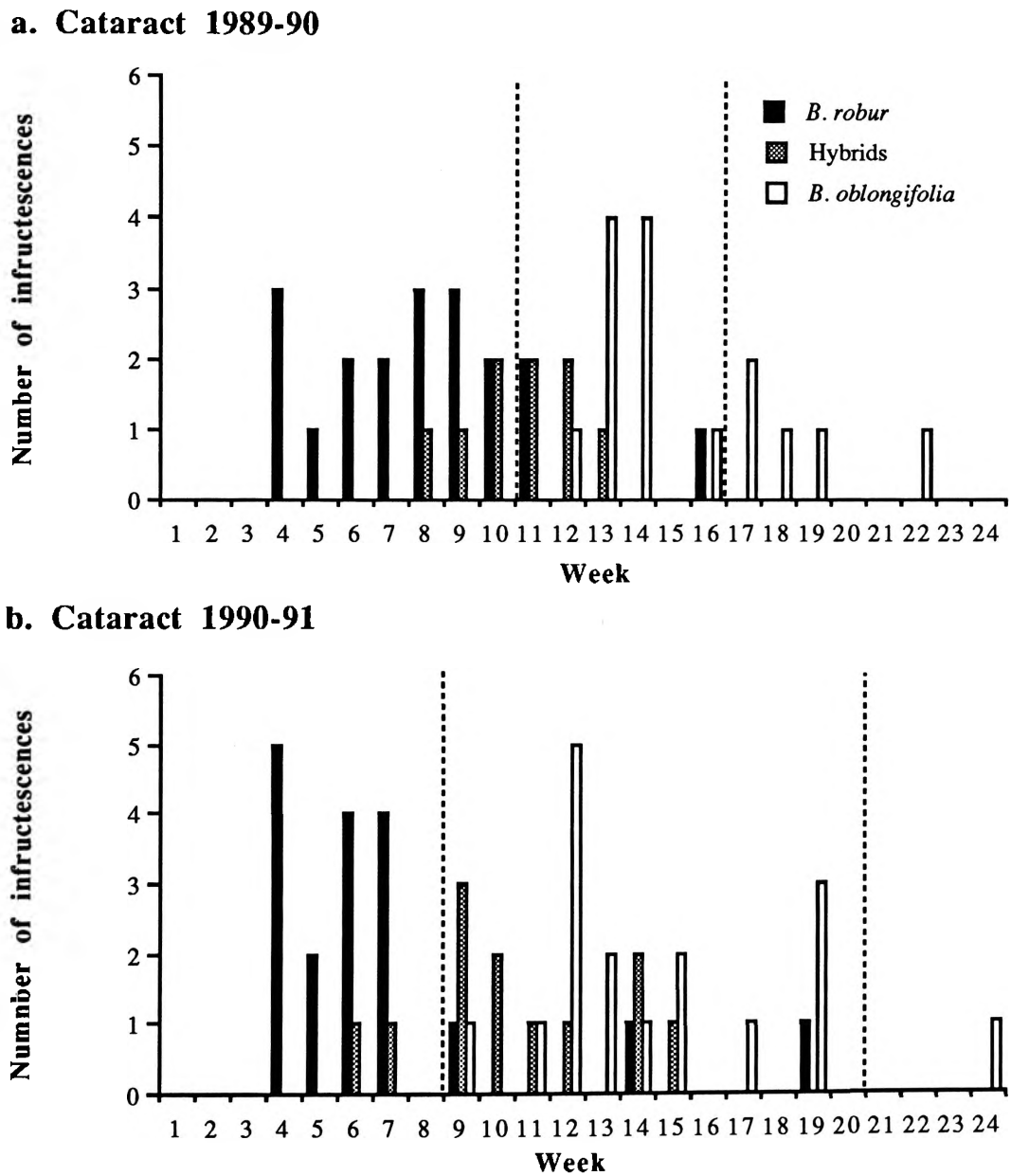
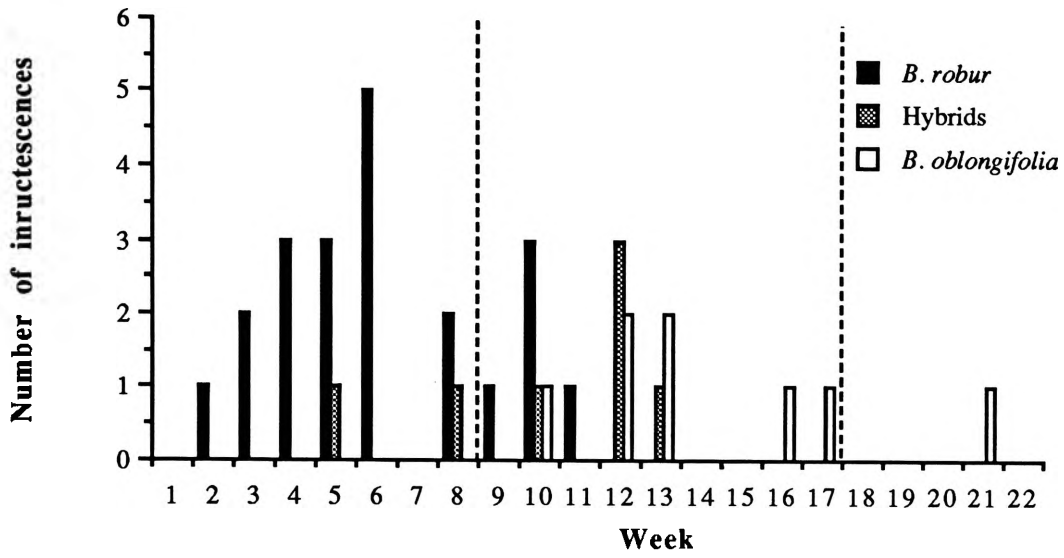
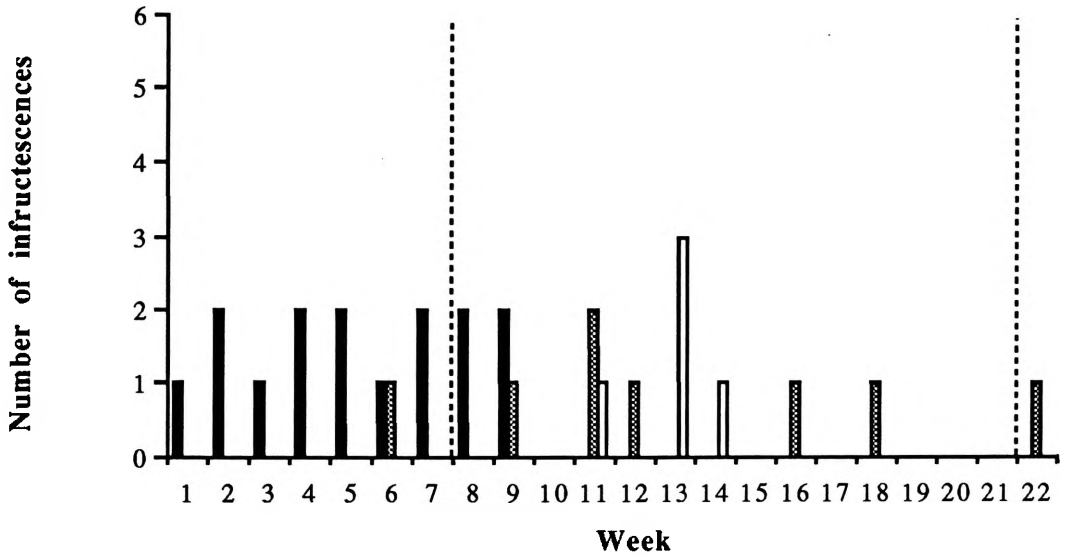


Figure 4.3. continued

c. Darkes Forest 1989-90



d. Darkes Forest 1990-91



oblongifolia was in flower. In the two populations, in the two seasons, 60% (Figure 4.3a), 100% (Figure 4.3b), 77.8% (Figure 4.3c) and 100% (Figure 4.3d) of the *B. oblongifolia* infructescences flowered while *B. robur* was in flower.

The hybrid inflorescences that eventually set fruit generally flowered during the time of overlap of *B. robur* and *B. oblongifolia* flowering. 74.2% of the hybrid infructescences flowered during the overlap of flowering of *B. robur* and *B. oblongifolia*, while 22.9% flowered during the hybrid/*B. robur* flowering overlap, and 2.9% during the hybrid/*B. oblongifolia* flowering overlap.

4.3.5. Potential for introgression

Figure 4.4 shows that for each parental morph, there is more chance of being pollinated by its own type than any other morph. Between 60.2 and 86.7 percent of the *B. robur* fruits produced in each population in each year were most likely to have been the result of pollination by *B. robur* pollen. Similar estimates were obtained from *B. oblongifolia* (between 60.1 and 88.3 percent). Expected pollination of the parental species in the hybrid zones by the hybrids varied greatly (between 6.8% and 39.2% for *B. robur*, and 10.5% and 29.3% for *B. oblongifolia*). However, it was slightly more likely for *B. oblongifolia* to be pollinated by *B. robur* than for *B. robur* to be pollinated by *B. oblongifolia*. The fruits produced by the hybrids were formed when there was potentially more or less an even proportion of pollen from all three morphs in the pollen pool.

4.4. Discussion

4.4.1. Flowering patterns of the three morphs

This study demonstrates that there is considerable potential for hybridization and introgression in the *Banksia robur/B. oblongifolia* hybrid zone, from the evidence of both flowering time and fruit set.

Figure 4.4. The estimated percentage of fruit produced as result of pollination by each morph in: a. Cataract 1989-90 season; b. Cataract 1990-91; c. Darkes Forest 1989-90; and d. Darkes Forest 1990-91. The bars represent the proportion of *B. robur* (R), hybrid (H) and *B. oblongifolia* (O) seeds pollinated by *B. robur*, hybrid and *B. oblongifolia* pollen.

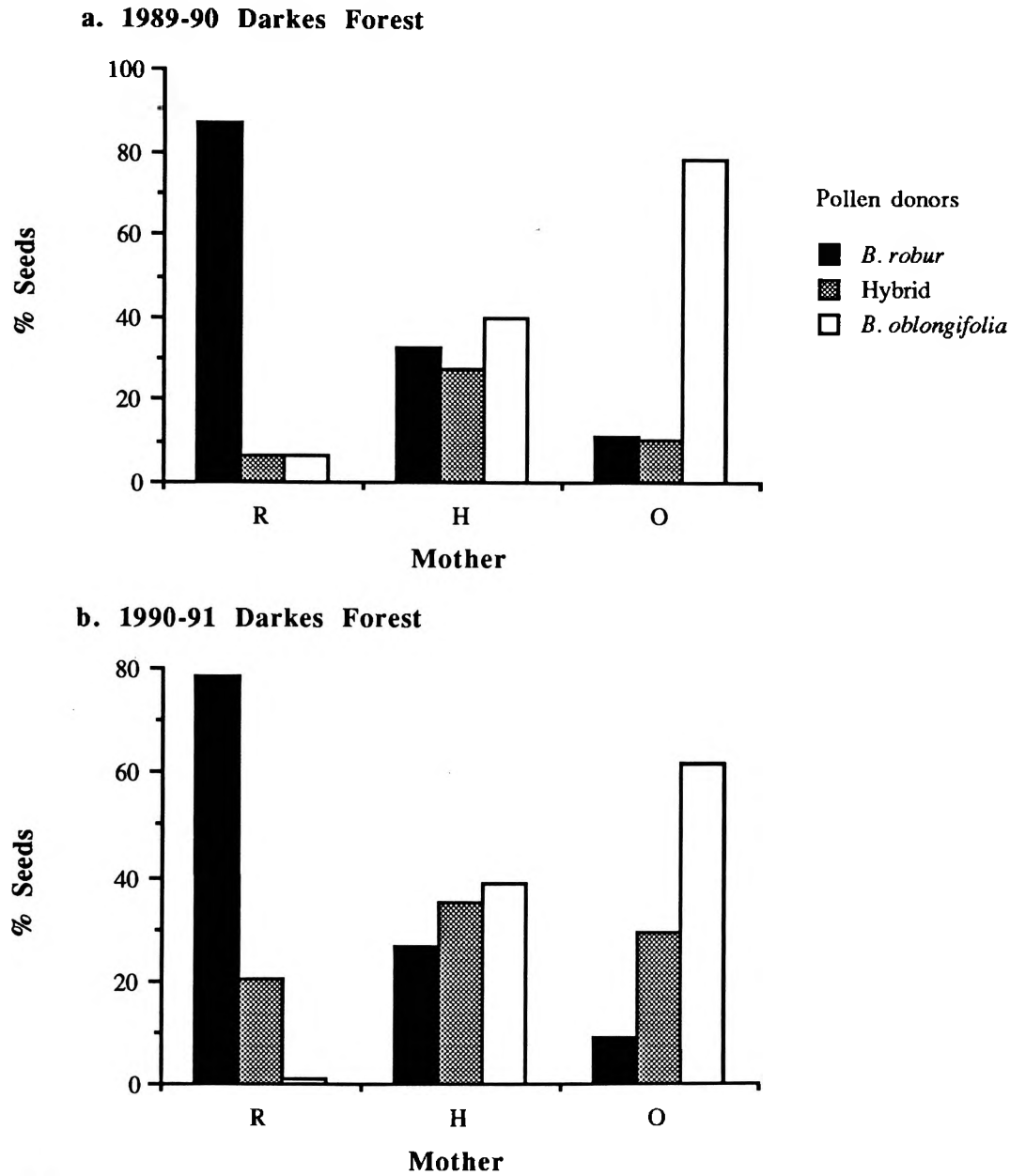
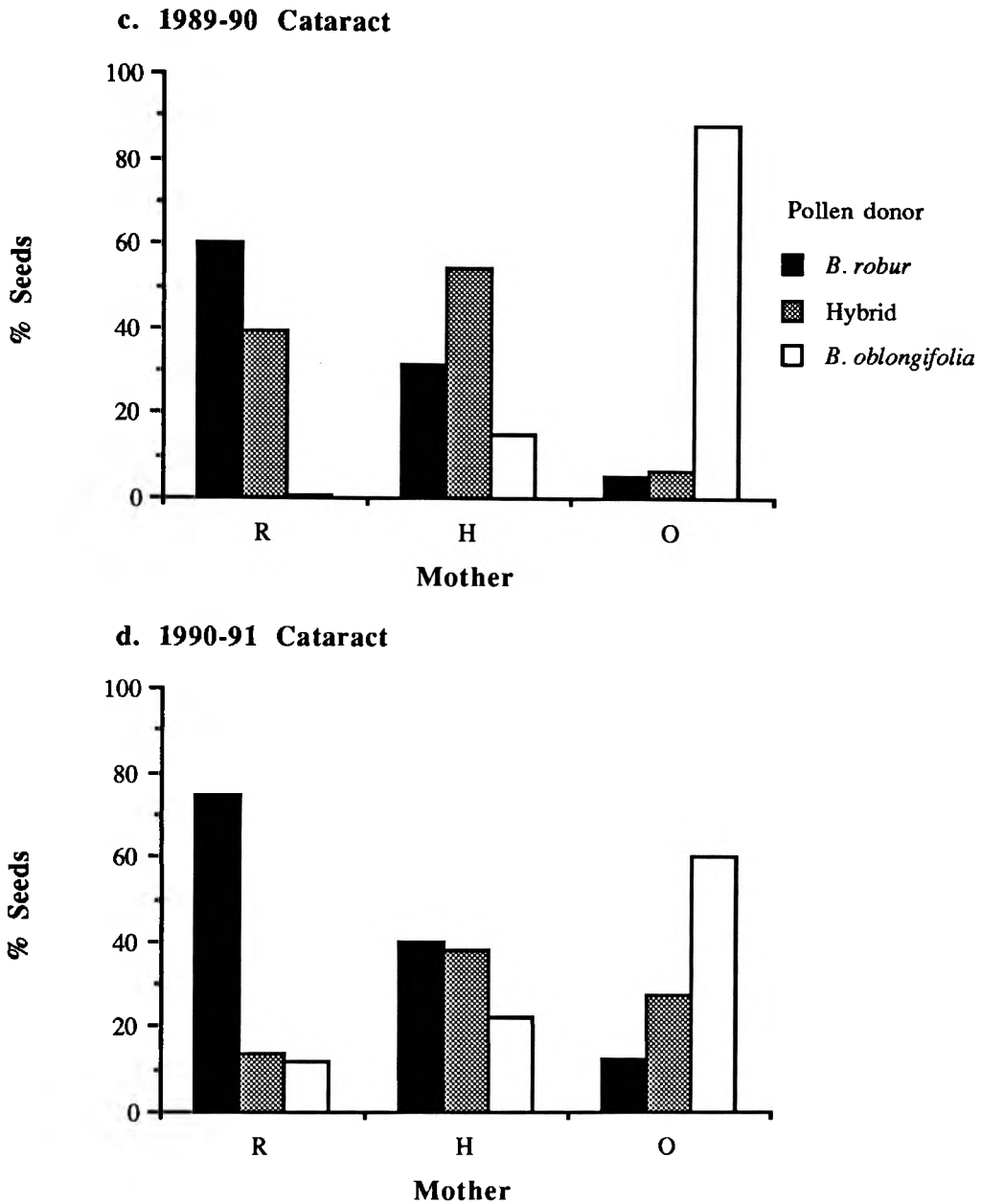


Figure 4.4. continued



There are clear differences in flowering time between the morphs. There was a separation of peak flowering time of the parental morphs within the hybrid zone, which was consistent between populations and between years. This consistency has been recorded in other species of *Banksia* (Copland & Whelan 1989). The flowering peaks of *B. robur* in the hybrid zone also corresponded closely to the peak observed in the pure stand of the species. However, a difference between the pure stand and hybrid zone *B. oblongifolia* was detected. This may indicate some shift in flowering time in this species.

Despite the clear separation of flowering peaks of the three morphs, there is the potential for gene exchange in two main ways. Firstly, a portion of the flowering time of the parental taxa coincided, providing some opportunity for interspecific pollination. The amount of overlap at the population level was consistent between years and between populations. Other published phenological studies on hybrid zones have shown variation in the degree of overlap in the parental species involved: from complete flowering synchrony (Drake 1980), to either some overlapping of the flowering times (Hopper and Burbidge 1978), or complete separation of flowering times (Drake 1980). This suggests that, without genetic information, it is necessary to determine the current flowering time, because the presence of hybrids in a population does not necessarily mean production of hybrids is still occurring. Secondly, and probably just as important in its implications for gene flow, the hybrid flowering time spans much of the flowering time of the two species, providing another channel of interspecific gene exchange through introgression. In fact, in some communities, contact between the two parent species is maintained only through the hybrid, as temporal (Drake 1980) and sometimes even spatial (Montanucci 1970) overlap no longer exists.

The limited amount of overlap in flowering time of parental taxa in this species complex may indicate different stages towards speciation or fusion of the gene pool of the species involved. The pattern of flowering observed in the *B. robur*/*B. oblongifolia* hybrid complex of parent 1 overlapping (but not completely synchronous) with hybrid

overlapping with parent 2 is what would be expected from a fairly recently formed hybrid zone (because differentiation or fusion of the species has not been complete), or one that is fairly stable (created by either a "tension zone" or hybrid advantage (Nichols and Hewitt 1988)). However, the sudden onset of flowering followed by a long tail, evident in the flowering time distributions, does not necessarily represent the beginnings of a shift in flowering time triggered by the coexistence of the two species. In fact, this result is commonly found in flowering distributions (e.g. Schmitt 1983a), and is more than likely a response to a regular, seasonal, environmental condition (Rathcke and Lacey 1985).

Skewness in flowering time could influence the amount of hybridization within the population, because the timing and duration of the period of flowering overlap can dictate the amount and direction of gene flow. The timing of onset and duration of flowering was consistent between populations and between years. *B. robur* was always the first to commence flowering in early summer. In both seasons studied, *B. oblongifolia* commenced flowering after *B. robur*'s flowering peak, while *B. robur* was always in flower during the *B. oblongifolia* flowering peak. This suggests there is the potential for asymmetry in the direction of gene flow between species (i.e. primarily from *B. robur* to *B. oblongifolia*), a conclusion that reinforces the directionality detected in the cline shape and introgression parameters calculated in Chapter Three. This directionality of gene flow is somewhat bridged by the intermediate flowering of the hybrids, the duration of which was such that there were hybrids in flower during both parental species flowering peaks in both years in both populations.

The skewness in the distribution of flowering time, however, may suggest that the overlap apparent at the population level is almost non-existent among individuals. This prediction of little real flowering overlap between species is confirmed when the overlap at the inflorescence level is considered. When a given inflorescence is open, the majority of other inflorescences in flower are of the same morph. The few inflorescences of the

other species open may provide little opportunity for interspecific pollination, but the majority of the gene flow between species could be primarily through the hybrid.

4.4.2. An example of reinforcement?

Despite the fact that the species are fairly well separated in their flowering time, this study does not present any convincing evidence so far that the *B. robur/B. oblongifolia* hybrid complex is undergoing reinforcement of reproductive isolation. Consistent shifts in peak flowering time, or the contraction of the duration of flowering of the species within the zone of contact, compared to allopatric populations, were not evident. Variation in these traits may be due to environmental or seasonal conditions, as discussed by Rathcke & Lacey (1985), and suggested by Butlin (1989) to be the real reason behind the variation in flowering time in other systems put forward as examples of speciation by reinforcement (e.g. McNeilly & Antonovics 1968). As there is no evidence for speciation by reinforcement for the *B. robur/B. oblongifolia* system, there is neither evidence excluding it. Longer term observation of flowering time is needed, as is the determination of the nature of the association of flowering time with microhabitat. Further reproductive isolation traits, along with the actual current hybrid production will be examined in the next two Chapters, and provide alternative characters where divergence is occurring.

4.4.3. Fruit set

Species within the family Proteaceae have chronically low fruit set (for discussion see Collins & Rebelo 1987 and Ayre & Whelan 1989), and the species in this study are consistent with this pattern (see Table 4.5). Conversion from inflorescence to infructescence in *B. robur, B. oblongifolia* and the hybrid is comparable to results obtained for other species in other studies (Whelan and Burbidge 1980; Copland and Whelan 1989). However, large differences in percent conversion between the populations suggests population specific differences (perhaps edaphic environment or pollinator activity) and emphasises the importance of studying more than one population before generalising about a species.

There has been considerable discussion about whether the timing of flowering within a community influences the number of seeds produced by individuals (Augspurger 1981, Gross and Werner 1983, Schmitt 1983a, Thomson 1985). Generally, there is a suggestion that the greatest seed set occurs in individuals with an intermediate flowering time, but this seems to vary between species (Gross and Werner 1983), or as result of environmental conditions (e.g. Thomson (1985) found low seed set in *Diervilla lonicera* individuals blooming during extended periods of rain). Differential seed production in individuals flowering at different times has been attributed to the attraction of pollinators to a larger or more dense flower show (Augspurger 1981).

This study demonstrated that inflorescences that set seed were those that flowered close to the flowering peak of each species. The most likely explanation is that pollinator foraging is more intense during the peak flowering times, although many other factors would come into play. For example, weather would affect pollinator activity, adhesiveness of pollen to stigma and successful maturation of seed. The distinct separation of the flowering times of the infructescences suggests that the amount of interspecific gene exchange may be small.

More evidence for the separation of the species is provided by the estimate of the proportion of each morph's pollen potentially in the pollen pool in the week of flowering of the inflorescences which eventually set fruit. The majority of fruits of each of the parental morphs were more likely to have been the result of intraspecific pollination. However, even when cross pollination was likely to occur, there is a suggestion of asymmetry in the amount of gene flow into the zone. *B. robur* has more opportunity to pollinate the *B. oblongifolia* than *vice versa.*

In this study, the well separated flowering times of the species are, therefore maintaining the genetic integrity of each species to a large extent.

The estimates obtained for the proportion of seeds of each species produced through pollination by intra-specific, inter-specific and hybrid pollen will be used to provide expectations to compare to the actual proportions produced through natural pollination, determined in Chapter Six.

4.4.4. Implications for gene exchange

Generally, the evidence from this study suggests that *Banksia robur* and *B. oblongifolia* tend to act as separate populations, even within the hybrid zone: separation of flowering peaks and ranges result in little opportunity for interspecific pollination to occur. Despite this, there are many intermediate individuals present in the population. This dilemma was noted in the Fire-bellied toad hybrid zone by Szymura and Barton (1986), in which different habitats were noted in the field and strong assortative mating was shown in laboratory breeding experiments.

The small amount of overlap of flowering between the two species is enough to produce hybrids within the population. This idea becomes more feasible when the *Banksia* life-cycle is considered. Most species of *Banksia* (including *B. robur, B. oblongifolia* and the hybrid [pers. obs.]) have the ability to resprout after fire, and the individuals are potentially very long-lived. Further, recruitment via seedlings occurs spasmodically (predominantly after fire), and even then may be low (pers. obs.). Continuation of the hybrid zone, therefore, may only require infrequent establishment of hybrids.

As the hybrid flowering time spans much of the flowering time of the parental species, and the fact that hybrids set a comparable amount of fruit to the parental species suggests that gene exchange between the parental species may be most frequently through the hybrid, and the complex is, therefore, gradually introgressing. In fact, introgression is the most likely outcome if the hybridizing species are fairly well separated reproductively (Stebbins 1959).

The results of this study suggest that the direction of gene flow between species seems to mostly in one direction: from *B. robur* to *B. oblongifolia.* Therefore, F_1 hybrids produced are more than likely to have a *B. oblongifolia* mother. This reinforces the suggestion in Chapter Three that the zone is essentially a "tension" hybrid zone (Hewitt 1988).

The flowering and fruit set phenology of *Banksia robur*, *B. oblongifolia* and their hybrid indicates that, per cohort, there is potentially a measurable amount of gene flow between the species and the hybrids. The apparent directionality of the gene flow indicates the beginning of the isolation of the gene pool, but even at this stage, the hybrid zone could be relatively stable, with constant production of hybrid individuals. This view, however, is complicated by the life-cycle of *Banksia*, because the generations may be long and overlapping. However, additional study of other stages where hybrid production may be stopped, such as observation of pollinators to determine the amount and any bias in the direction of pollen flow, and the determination of post-mating processes, which will be attempted in the following two Chapters, will contribute to the determination of selective processes within these complexes.

Chapter Five

Pollen dispersal within the hybrid zone.

5.1 Introduction

The formation of hybrids between plant species not only requires the coincidence of flowering, but the successful transfer of pollen from one species to stigmas of another, and the fertilization of the ovule by the transferred pollen. The existence of hybrid and backcrossed plants within the two hybrid zones under study indicates that substantial interspecific pollen transfer has taken place between *Banksia robur* and *Banksia oblongifolia* in the past, but may not indicate that hybridization is currently occurring. Chapter Four showed that the species flowered simultaneously within limited periods, providing the current opportunity of the interspecific pollen transfer. This Chapter presents evidence of the potential for pollen transfer between the species and the distance over which this transfer may be effected.

5.1.1. Effects of pollen movement on population structure

The pattern of movement of genes within a population can influence its genetic structure. Limited dispersal of genes is thought to result in a genetically structured populations, with genetically related individuals mating with each other more commonly than would be expected at random, while, in contrast, wide gene dispersal is assumed to create a genetically homogeneous populations (Wright 1978). This is especially thought to be so in sessile organisms such as plants, where gene dispersal is primarily mediated through vectors, which transfer pollen and seed (Levin & Kerster 1974). Therefore, gene flow in plants is typically fairly restricted (Levin 1981, Hamrick 1982). Indeed, studies on the the dispersal distances of pollen have often found to be leptokurtic (Wyatt 1983, Loveless & Hamrick 1984, Bos *et al.* 1986). Leptokurtic pollen dispersal in plant populations is found among dispersal via different pollen vectors (biotic and abiotic) (Loveless &

Hamrick 1984), although the scale of this movement is very much dependent on the vector (Waddington 1981, Waser 1988, Peakall 1989).

The effect of gene flow on the level of genetic structure within a population was considered by Wright (1943, 1946) in his "neighbourhood" model. A measurement of the homogeneity of the population is through the determination of the genetically effective neighbourhood size: both the area of the neighbourhood it occupies and the number of breeding individuals it comprises (effective population size). When neighbourhoods are small, genetic differentiation may take place even if the population is continuous, as any dispersal that occurs will tend to be swamped by local fixation (Cahalan & Gliddon 1985). In the absence of selection, Wright (1946) considered that effective populations of less than 200 will exhibit differentiation, and when the neighbourhood size is less than twenty, this differentiation will be considerable.

5.1.2. Pollen dispersal within a hybrid zone

Within a contact zone between two different species, the extensive, long distance pollen dispersal may cause the species to become increasingly genetically similar, perhaps resulting in the convergence of some traits. For example, Sanderson (1989) suggested that gene flow will swamp the formation of the reinforcement of reproductive isolation that has arisen between the species, with the proviso that gene flow (i.e. gene dispersal AND establishment) has occurred. Restricted gene flow among near neighbours, which are most likely to be of the same species, will reduce the amount of hybridization that will occur in the contact zone. If interspecific pollinations commonly occur, the rate and scale of this gene flow will be important determinants of the gradient of the cline (Szymura & Barton 1986 and determined in Chapter Three). Therefore, foraging behaviour of pollinators within a hybrid zone can potentially dictate the ultimate stability of the hybrid zone (Endler 1977).

5.1.3. Pollinator behaviour within a mixed species stand

If animals are necessary as pollen vectors, the pattern of foraging used by the potential pollinators visiting flowering plants is important to the pattern of pollen movement within the population under study (Linhart *et al.* 1987). A range of studies has shown that pollinators vary widely in their faithfulness to plant species. While some plants have evolved with very specific pollinators, particularly where the attraction is a pseudo-sexual attraction, there are actually few documented cases in which pollinators forage exclusively in one type of plant (Fægri and van der Pijl 1979). Therefore, when two or more sympatric species flower simultaneously, there will often be some transfer of pollen between species. If this occurs, it is thought that the transfer of pollen from one species to another (for which Rathcke (1983) coined the phrase "improper pollen transfer") may have greater selective force than some other factors, such as competition for pollinators, in forming and maintaining mate recognition systems (Waser 1982). Separation of flowering time (Mosquin 1971) and differentiation of floral characters (Waser 1983), which are important specific mate recognition characters amongst plants, are some consequences that have been attributed to improper pollen transfer. The selection against individuals produced by improper pollen transfer (i.e. hybrids) may be strong enough to overcome the homogenizing force of gene flow (Sanderson 1989). Even if interspecific pollination does not result in the less fit individuals, the establishment of hybrids in a population may help to maintain the differences between the species, by acting as gene sinks (Barton 1979), because matings between pure species will become rarer, as the hybrid population grows.

5.1.4. Pollination of *Banksia*

Floral visitors to *Banksia* include birds (honeyeaters), small mammals (e.g. native rats, antechinus, pygmy possums, sugar gliders) and insects (bees (introduced and native), ants, flies). Studies of pollination in *Banksia* have suggested that birds are important pollinators in most species (Paton & Turner 1978; Ford *et al.* 1979; Hopper 1980; Whelan & Burbidge 1980; Collins & Spice 1986; Collins & Rebelo 1987; Ramsey 1989;

Vaughton 1992). Many studies have observed that honeyeaters are generalist foragers and opportunists (Ford *et al.* 1979). Honeyeaters will stay in an area where nectar is abundant (which can be year round in certain plant assemblages [Whelan & Burbidge 1980]), although their abundance is not always correlated with the amount of nectar available (Pyke 1988).

Pollination of *Banksia* flowers is thought to be more consistently performed by honeyeaters rather than any of the many insect foragers. Birds mostly forage around the region of newly open flowers, which produce the most nectar. Therefore, these flowers have the highest chance of pollen, carried by the bird, being deposited on the pollen presenter (Collins & Spice 1986). Pollen is often concentrated on the forehead, chin feathers and beak of mist-netted honeyeaters (Ford *et al.* 1979), which are also the parts of the bird that will subsequently come in contact with stigmas. In addition, birds may be more reliable and constant pollinators than insects when flowering seasons are unpredictable or during winter (Ford *et al.* 1979). This was confirmed by Vaughton (1992), who found that birds visited *B. spinulosa* throughout the flowering season (which included the winter months), while bees visited only on warmer, spring days.

There has long been a suggestion that mammals play some role in the pollination of many Australian plants (Rourke & Wiens 1977; Collins & Rebelo 1987), and there have been several attempts to assess the importance of mammals as pollinators of *Banksia* (Carpenter 1978, Hopper 1980, Goldingay *et al.* 1987, Cunningham 1991, Carthew 1993a). Most of these studies concluded that non-flying mammals may be involved in some pollen transfer. Mammals are also regarded as "unfaithful" pollinators, feeding in up to three species of *Banksia* in the one foraging bout (Carthew 1993a). Therefore, non-flying mammals may also be expected to be interspecific pollen vectors.

5.1.5. Inferred pollen flow distance

The distance a potential pollinator travels between flowers or inflorescences on which it feeds can only be considered the "potential pollen flow distance" (Levin & Kerster 1974). The successful transfer of pollen by vectors is determined by other factors, such as the pollinator's anatomical suitability to transfer pollen successfully within a particular species (Ford *et al.* 1979) and pollen carryover (Schaal 1980, Waser & Price 1984). Indeed, study of *Eucalyptus stoatei* revealed that genetically determined level of outcrossing was far higher than the estimate obtained by the study of pollinator movements (Hopper & Moran 1981).

Previous studies have estimated the movement of pollen within a population by using pollen substitutes, such as dyes (e.g. Webb & Bawa 1983, Waser 1988, Campbell & Waser 1989). Again this technique can only measure the distance pollen may be transferred, and not the success of the pollinations. A more accurate estimate of pollen flow can be made using genetic markers (Handel 1982, Smyth & Hamrick 1987). The actual distance pollen has been transferred is a major component of gene flow within a plant population (Campbell & Waser 1989), and the distance of pollen dispersal can be reliably estimated from the distance a rare allele has been dispersed from its source, and established in the genome of another section of the population (Slatkin 1975) (assuming, of course, that the allele is selectively neutral). Many studies estimating the scale of pollen flow using genetic markers have been conducted within artificial situations (plantations in Smyth & Hamrick [1987], and regularly spaced plants within experimental gardens in Handel [1982] and Campbell & Waser [1989]), making it easier to control the source of the rare allele. This control is not possible within natural plant populations, but there have been studies attempting to describe gene flow combining the observation of pollen flow, pollinator movements and genotyping seed (e.g. Schaal 1980, Hopper & Moran 1981, Campbell 1991).

This Chapter aimed (i) to determine the pollen vectors visiting the inflorescences of *Banksia robur* and *B. oblongifolia* and their hybrid, and (ii) to assess the relative importance of floral visitors in the transfer of pollen between the two species, and between the species and the hybrid. The distance pollen was transferred by the potential pollinators was compared to the inferred pollen movement distance, determined using the distance dispersed by a rare allele within the population. These distance measurements were used to determine effective population size and neighbourhood area, and the results compared to the gene dispersal distance obtained in Chapter Three using cline shape parameters.

5.2. Methods

5.2.1. Bird Foraging Observations

Foraging of birds was observed within the Darkes Forest hybrid zone, when both species were in flower (between March and April). Observations were made using binoculars, from a step ladder, which was placed near a tree or tall bush, to minimise the conspicuousness of the observer. Observations of each foraging bout began when a bird landed to feed on a plant within the observation area and ended when the bird either perched on the bough of a tree or flew out of sight, although it was possible that foraging occurred before and after the recorded movements within the bout. The morph of the each inflorescence in which the bird foraged and the distance travelled between inflorescences on which the bird foraged was recorded for each observed bout. The proportion of each type of morph visited was compared to the proportion of flowering inflorescences of each morph within the site using chi-square analysis.

The mean distance travelled by each species separately, and the total over all species were both determined, because different feeding patterns of different species of bird could result in disparate pollen dispersal patterns. Significant differences in the total distance flown within a foraging bout and the distance flown between subsequent inflorescences for each species of honeyeater were determined using Mann-Whitney U tests.

5.2.2. Mammal Trapping

Trapping was conducted when both species of *Banksia* and the hybrids were flowering (i.e. the end of March to the beginning of April [see Chapter Four] in the years 1991, 1992 and 1993). Traps were set only near those plants which had inflorescences secreting nectar, so there was not necessarily an equal number of traps set near inflorescences from each morph each trapping night. Trapping methods followed Goldingay *et al.* (1987).

Mammals were trapped in Elliott traps (100mm X 100mm X 300mm), which were placed so that the entrance to the trap was a few centimetres from an inflorescence that was secreting nectar. This was achieved by securing the traps in a piece of P.V.C. pipe cut down one side, which was fastened to a 1 metre high stake using wire, so that the height of the trap could be adjusted. Each trap was then baited with a small amount of creamed honey, which was replenished after each night of trapping.

Traps were left overnight on all occasions. On some of these occasions, traps were checked at night, after they had been set for about 5 hours, as well as in the morning. The species of animal, and the morph of the nearest inflorescence were noted.

In order to measure the movements of the animals while foraging, spool-and-line tracking (Carthew 1991) was attempted. This technique involves the fastening a spool of thread covered with a layer of electrical tape to the animal's back, using a small amount of instantaneously drying glue. One end of the thread was then fastened to the vegetation, and the animal was released. The thread then unravelled as the animal travelled. An advantage to this technique is that the line, and thus movements of the animals, can be followed some time after the animals had been caught and spooled.

All attempts to attach spools to animals failed. In all but one instance, the spools fell off immediately with moulted fur, when the animals brushed against the vegetation on

release. The only spool that didn't fall off immediately was traced for about thirty metres before it fell off. This animal did not forage on *Banksia* within the thirty metres.

5.2.3. Pollen dispersal distance and direction

The realized distance of gene flow within the hybrid zone was estimated using the non-specific dehydrogenase locus, determined from the NSdh locus (detected in the electrophoretic survey in Chapter Two). A rare allele on this locus, the s allele, was found in a few plants as heterozygotes only with the f allele. The positions of these plants were determined using Figures 3.1 and 3.2. The presence of the s allele in the progeny array of plants that were homozygous for the f allele, indicated the dispersal of the pollen from the plants with the alternative allele. Comparison of the progeny 4-locus genotype with that of the plants with the heterozygote genotype at the NSdh locus enabled the identity of the male parent to be determined. The proportion of the progeny array from the infructescence that were potentially sired by identified pollen source plant was also calculated. This proportion was plotted against the distance of the seed plant from the pollen source plant, to determine the pattern of pollen dispersal.

The position of the pollen donor and recipient plants was mapped and the distance between them recorded. The mean distance dispersed by the allele and its variance was determined. The plants used in this analysis were assigned the hybrid index scores to determine if there were any detectable patterns of dispersal between parental species, F_1 and F_2 hybrids.

5.2.4. Pollen /gene dispersal and neighbourhood size

Three estimates of pollen and gene dispersal are available for comparison: the distance flown by bird pollinators, the distance dispersed by the marker allele, and the dispersal variance estimated in Chapter Three from the cline width.

These dispersal distance variances were used to determine the genetic neighbourhood area (N_A) and the neighbourhood population size, sometimes referred to as the genetically effective population size (N_e) (Cahalan & Gliddon 1985, Bos *et al.* 1986), using the formulae:

$$N_e = 2 \pi t d ([\sigma^2_p + \sigma^2_s] / 2)$$

and

$$N_A = 4 \pi ([t \sigma^2_p / 2] + \sigma^2_s)$$

where t is the outcrossing rate, d is the density of reproductively mature plants (genetically effective density), σ^2_p is the variance of the dispersal distance of the pollen, and σ^2_s is the variance of the seed dispersal distance. The seed dispersal distance for other species of *Banksia* has been shown to be negligible (Abbott 1985), and so the term will be excluded from the calculations. The outcrossing rate will be determined for this system in Chapter Six, so all estimates of N_A and N_e will be multiplied by t.

Schmitt (1980) used an expanded version of the formula determining the neighbourhood size, taking into account the contributions of the different pollinators:

$$N_e = 2 \pi t d ([as^2_A + (1-a) s^2_B + \sigma^2_s] / 2)$$

where a is the proportion of pollen transfer by pollinator A, (1-a) is the proportion of pollen transfer by pollinator B, s^2_A and s^2_B are the observed flight distance variances for pollinators A and B respectively. This formula will be used to determine the neighbourhood size using the potential pollinator flight distances, and will used as a comparison to the results obtained using the marker allele.

5.3. Results

5.3.1. Bird Foraging

In a total of 44 hours of observation, four species of honeyeater were observed foraging: the red wattlebird, *Anthochaera carunculata*, the New Holland honeyeater, *Phylidonyris novaehollandiae*, the Eastern Spinebill, *Acanthorhynchus tenuirostris*, and Lewin's Honeyeater, *Meliphaga lewinii* [identified using Pizzey (1980) and Slater *et al.* (1989)]. Foraging was dominated by the New Holland honeyeater and the red Wattlebird, as only three foraging bouts were observed for the Spinebill and only one for the Lewin's.

A total of 45 flights between inflorescences by the red wattlebird were observed (Table 5.1). The most flights observed were between the inflorescences of different plants of the same morph (20), although there were 19 flights between two or more inflorescences on the same plant. In contrast, the New Holland honeyeaters spent most of the time foraging in inflorescences on the one plant, while just over a quarter of the total flights observed were between plants of the same morph. Most of the between morph flights of the New Holland honeyeater and the wattlebird (and the between morph flights observed for the Spinebill and the Lewin's) were from a *B. robur* inflorescence to a *B. oblongifolia* inflorescence. A statistical comparison was conducted between the wattlebird and New Holland honeyeater only, because these species were the most common visitors to these species of *Banksia*. There was no significant difference between the wattlebird and the honeyeater in the number of inflorescences visited per bout (Mann-Whitney U: $U'_{43,130}=3323$; $p>>0.5$) (Table 5.1).

There were significant differences detected between the proportions of each morph in flower and the proportion of morphs visited by both the New Holland honeyeater and the red wattlebird (Table 5.2). In both cases significantly less hybrid and significantly more *B. robur* inflorescences were visited than would be expected if inflorescences were visited at random.

Table 5.1. The number of foraging bouts observed within a single inflorescence on a single plant (Single Plant), and between inflorescences on different plants (Between Plants), for the four species of bird observed foraging within the hybrid zones. R are *B. robur* plants, H are hybrid plants and O are the *B. oblongifolia* plants. The combinations of R/O, R/H, O/H and R/H/O indicate the observed instances of birds flying between *B. robur* and *B. oblongifolia*, *B. robur* and hybrid, etc., in any order of visitation.

	Red Wattlebird	New Holland Honeyeater	Eastern Spinebill	Lewin's Honeyeater	Total
Single Plant					
R	13	55	1	0	**69**
H	0	2	1	0	**3**
O	6	16	2	0	**24**
Between Plants					
R	14	19	0	0	**33**
H	0	0	0	0	**0**
O	6	17	2	0	**25**
R / O	5	17	1	1	**24**
R / H	0	2	0	0	**2**
O / H	0	2	0	0	**2**
R /H / O	1	0	0	0	**1**
Total	**45**	**130**	**7**	**1**	**183**

Table 5.2. Contingency table analysis to determine if there is bias of the bird species to visiting a particular type of *Banksia*. The observed figures were the number of visits to the type of inflorescence, while the expected frequencies are based on the proportion of each type of inflorescence open at the time. The observations for the two predominant bird foragers only were used a. Contingency table for the New Holland honeyeater, b. for the Red wattlebird.

a. New Holland honeyeater

Inflorescence visited	Observed	Expected
B. robur	125	105.12
Hybrid	5	21.90
B. oblongifolia	89	91.98

χ^2=16.897 p<<0.001

Conclusion: More *B. robur* and less hybrids visited than expected, no difference between expected and observed for *B. oblongifolia*

b. Red wattlebird

Inflorescence visited	Observed	Expected
B. robur	57	41.28
Hybrid	1	8.6
B. oblongifolia	28	36.12

χ^2=14.528 p<0.001

Conclusion: More *B. robur* and less hybrids and *B. oblongifolia* visited than expected.

The foraging birds generally flew short distances between successive plants. Most foraging flights for all three species of bird were between 1 and 4 metres (Figure 5.1). The mean distances flown between successive plants for each species of bird (excluding the Lewin's honeyeater, for which only one observation was made) was about 10 metres, the average of the total observed flights being 10.6 (Table 5.3). This confirms the interpretation of the frequency distribution (Figure 5.1), which suggested that the flights between successive inflorescences was fairly small, but not necessarily between adjacent plants. There was no statistically significant difference between the distances travelled between plants by the wattlebirds and the honeyeaters (Mann-Whitney U: $U'_{46,99}=2534$; $p>>0.5$). Distances travelled between successive inflorescences varied depending on the combination of types visited (Table 5.4). Predictably, the average shortest distance travelled was between like inflorescences (from *B. oblongifolia* to *B. oblongifolia* [7.4] and from *B. robur* to *B.robur* [8.3]), and the longest from *B. oblongifolia* to *B. robur* (23.3) and *B. robur* to *B. oblongifolia* (18.1).

The total distance covered within a foraging bout for all species of birds did not exceed 79 metres (Figure 5.2) Indeed, the mean distances travelled for all four species of bird were fairly short, the New Holland honeyeater having the longest mean (except for the one observation for the Lewin's of 50 metres) at 19.1 metres. The average for all bouts observed was 18.6 metres. Again there was no significant difference detected between the wattlebirds and honeyeaters in the distance travelled within a foraging bout (Mann-Whitney U: $U'_{27,55}=786$; $p>0.5$).

5.3.2. Mammal Trapping

The only species of mammal caught was the brown antechinus, *Antechinus stuartii*. In a total of 258 trap nights, 12 animals were caught, giving a trapping success of 4.7%. These animals were caught in all three morphs of *Banksia*, but with different success (Table 5.5). The greatest trap success was near *B. robur* inflorescences, while fewer animals were caught near *B. oblongifolia* inflorescences.

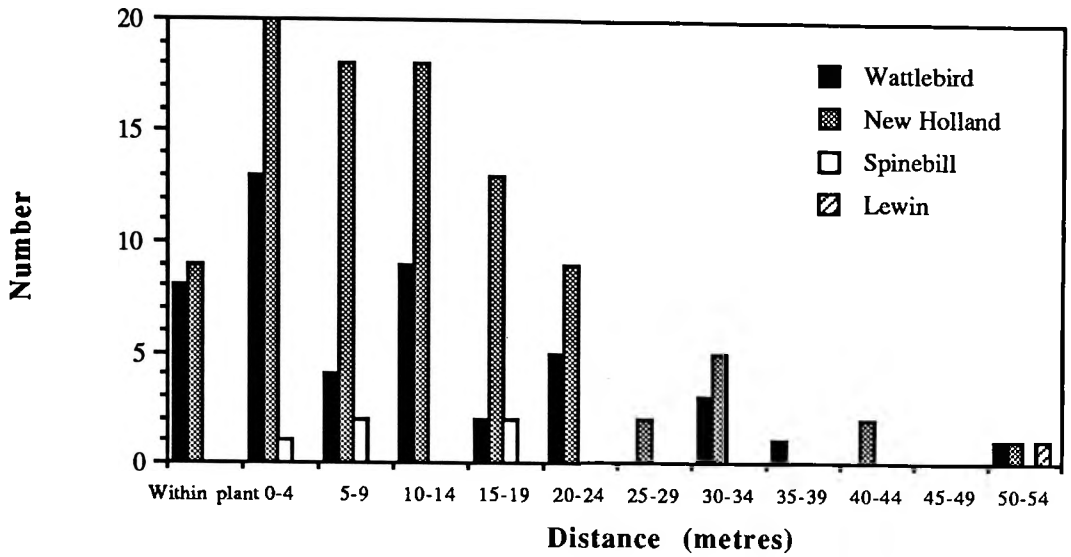


Figure 5.1. Frequency distribution of the distance flown between successive inflorescences visited, for the four species of birds observed foraging within the hybrid zone. "Within plant" category indicates that more than one inflorescence was visited within the one plant

Table 5.3. Mean distances covered between successive inflorescences in which foraging occurred, and over the entire foraging bout, for each of the three species of bird observed foraging in the hybrid zones.

	Red wattlebird	New Holland honeyeater	Eastern spinebill	Lewin's Honeyeater	Total
Between Inflorescences	10.0	10.6	9.0	50	10.6
(±S.E.)	(±1.7)	(±0.1)	(±2.4)	-	(±0.9)
N=	**46**	**99**	**5**	**1**	**151**
Within Bout	16.6	19.1	15	50	18.6
(±S.E.)	(±2.8)	(±2.1)	(±0.0)	-	(±1.7)
N=	**27**	**55**	**3**	**1**	**86**

Table 5.4. Mean distance (± standard error) flown between successive inflorescences for the Red wattlebird and the New Holland honeyeater. No standard error is shown for those categories for which only one bout was observed. Categories with a dash indicate no observation was made. O = *B. oblongifolia* inflorescences, H= hybrid inflorescence and R=*B. robur*.

	Red Wattlebird	New Holland Honeyeater	Spinebill	Total
O-O	8.8 (±3.1)	7.1 (±1.0)	7.5 (±2.4)	7.4 (±1.0)
O-H	-	-	-	-
O-R	13.3 (±2.7)	23.8 (±5.4)	-	23.3 (±4.5)
H-O	-	12.5 (±1.8)	-	12.5 (±1.8)
H-H	-	-	-	-
H-R	1	10	-	5.5 (±3.2)
R-O	13.4 (±3.0)	20.2 (±8.2)	15	18.1 (±2.6)
R-H	5	11.5 (±2.5)	-	9.3 (±2.4)
R-R	8.4(±2.4)	8.2 (±1.3)	-	8.3 (±1.3)

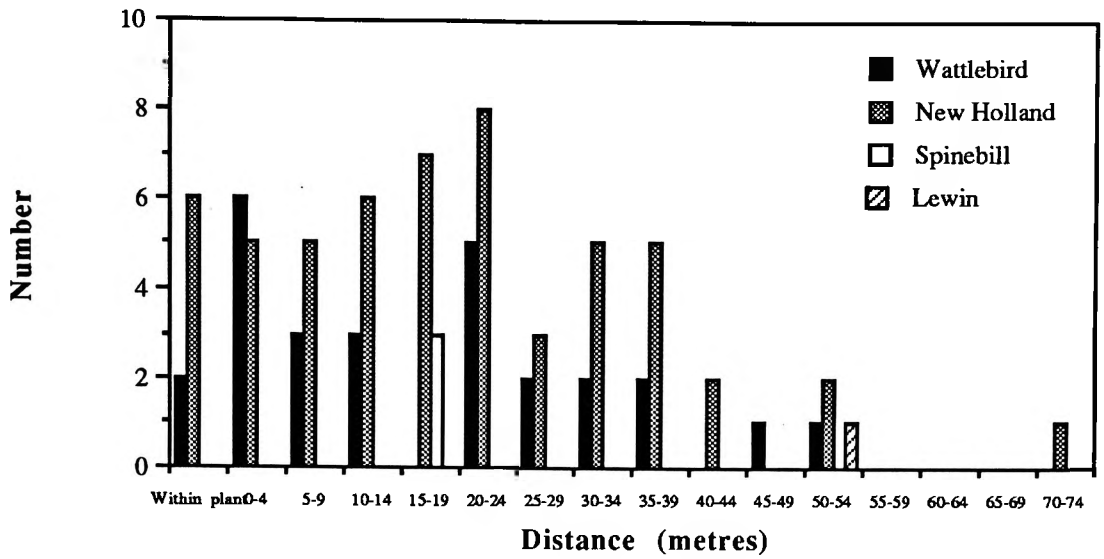


Figure 5.2. Frequency distribution of total distance covered within a foraging bout for the four species of bird observed foraging within the hybrid zone. "Within plant" category indicates that the entire bout consisted of visits to more than one inflorescence on the one plant.

Table 5.5. Numbers of *Antechinus stuartii* captured within parental species and hybrid. Trap success is the percentage of captures per trap night.

	Trap Nights	Captures	Trap Success (%)
B. robur	100	7	6.0
Hybrid	50	4	8.0
B. oblongifolia	108	1	0.9
Total	258	12	4.7

5.3.3. Pollen dispersal

The average distance pollen was dispersed, according to the dispersal of the s allele on the non-specific dehydrogenase, was 8.33 (±0.78) metres in the Cataract population and 10.87 (±1.98) in the Darkes Forest (the overall mean was. The frequency histogram of the distance the pollen travelled from the source (Figure 5.3) shows that the most frequent distance travelled from the pollen source plant is about six metres. The proportion of seeds sired by a particular plant dropped away rapidly with distance, forming a leptokurtic distribution (Figure 5.4).

The genetic hybrid index score (GHIS) of the plants heterozygous for the s allele on NSdh was recorded (Chapter Two). Pooled over both populations, there were three plants each that had a GHIS of 3 and 5, while two plants had GHIS of 2, 4 and 6. Most of these plants sired seed on plants with GHIS of 3, 4, 5 and 6 (Figure 5.5). Only one *B. robur* plant was pollinated by a plant with the s allele on the NSdh.

5.3.4. Neighbourhood area and size

The two different methods for determining the gene dispersal variance yielded variable estimates of the neighbourhood area and the number of plants within the genetically effective population (Table 5.6). The estimates of σ^2, N_e and N_A obtained using the marker allele were very small, but the magnitude of these parameters between populations were similar. The effective population size for both populations was between one and two individuals, while the neighbourhood area was in the order of 100 to 200 m^2. The estimates obtained from the distance flown by birds were larger than those obtained using the marker allele. For flights between inflorescences, the effective population size was around six individuals, while the neighbourhood area covered around about 800m^2. There was not a great difference in the estimates of σ^2, N_e and N_A between the distance flown between inflorescences and the distance flown in an entire bout. The effective population size increased to 11, while the neighbourhood occupied an area of 1500m^2.

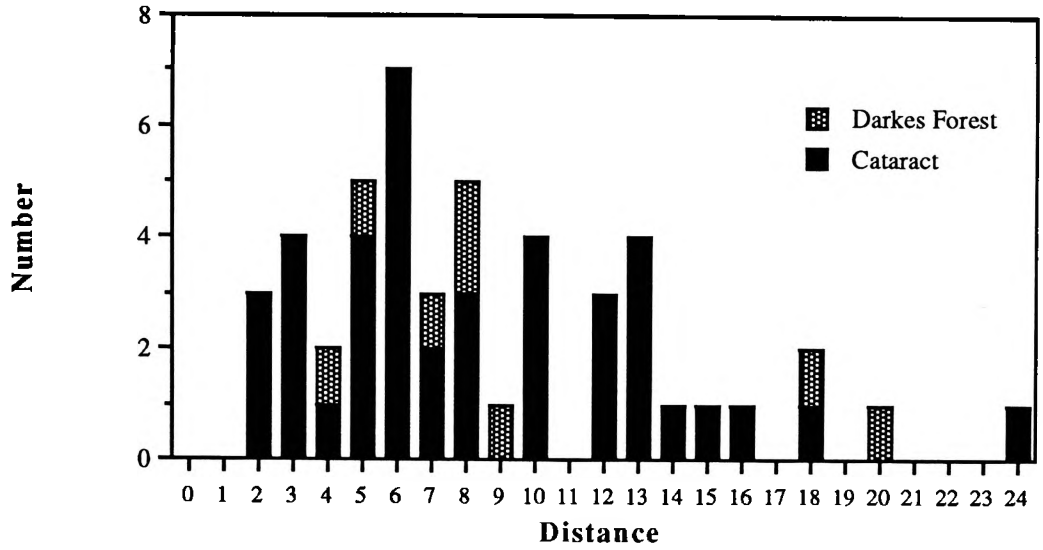


Figure 5.3. Frequency distribution of the distance of pollen dispersal from the source plant, determined using the s allele on the Non-specific Dehydrogenase Locus (NSdh). The measurements for both populations are shown.

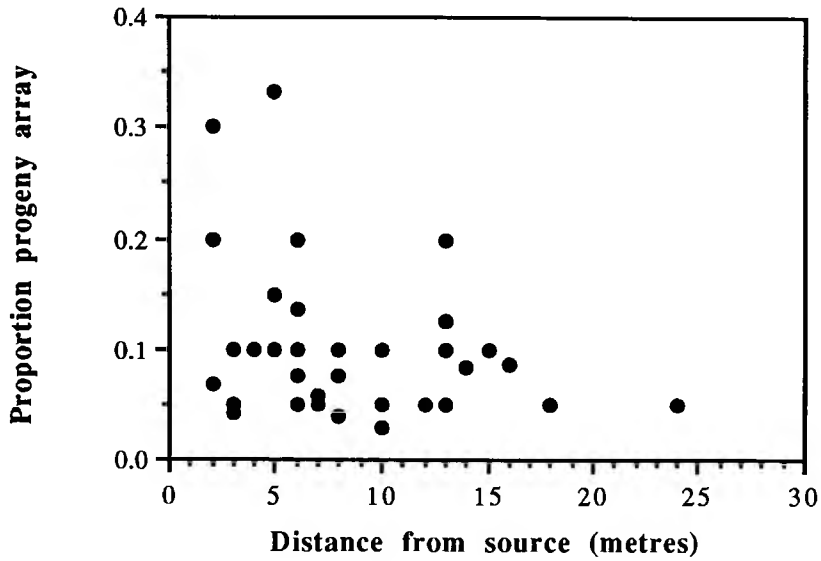


Figure 5.4. The plot of the proportion of progeny sired by a plant against the distance from that plant, determined using the s allele on the Non-specific Dehydrogenase Locus (NSdh).

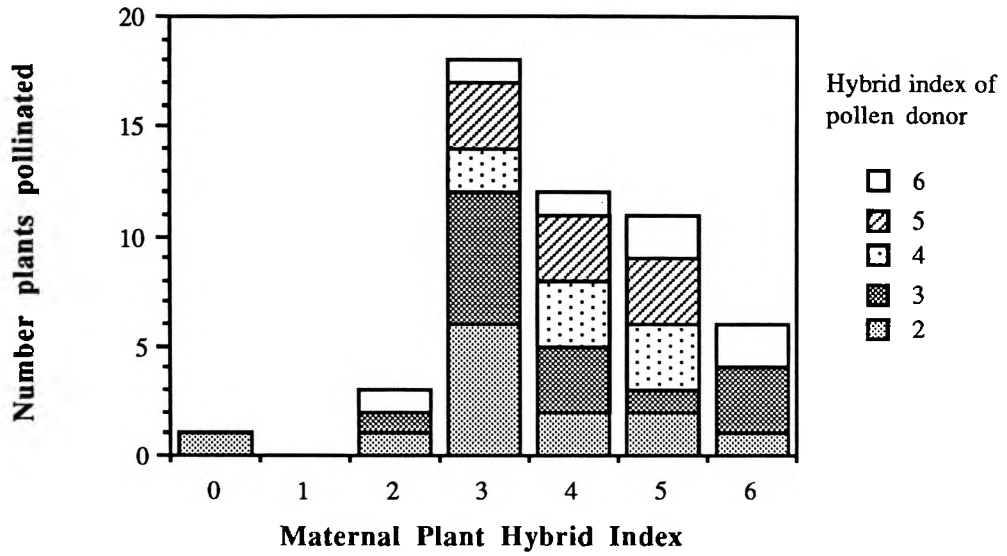


Figure 5.5. Comparison of the genetic hybrid index scores of the seed and siring plants. The siring plants were all heterozygous for the s allele on the Non-specific Dehydrogenase Locus, and were able to be accurately identified using their four-locus genotype. No plants with GHIS of 0 or 1 were heterozygous for the s allele on the NSdh.

Table 5.6. Estimates of the variance of gene dispersal distances (σ^2), effective population size (N_e) and neighbourhood area (N_A), using a. the distance travelled by birds between inflorescences and within an entire bout and b. using the estimated distance dispersed by the marker allele (the NSdh s allele).

	σ^2	N_e	N_A
a. Birds			
Between inflorescences	122.31	6.15	768.50
Within Bout	248.54	11.27	1561.62
b. Marker allele			
Cataract	25.29	1.27	158.90
Darkes Forest	35.21	1.77	221.23

5.4. Discussion

The results presented in this chapter suggest that the transfer of pollen between parental species, and from the parental species to intermediate plants and vice versa, occurs frequently. None of the foragers observed appeared "faithful" to any one of the morphs under study: all major types of visitors observed, foraged in both the parental species and the hybrid. It seems that these two species, like many other species of *Banksia*, have a "combined pollination system" (Goldingay *et al.* 1991) in which many types of pollinators forage at plants and are capable of successfully pollinating the flowers.

5.4.1. Floral characters and potential pollinators

The evolution of many plant features have been attributed to the attraction of a certain suite of pollinators or the inevitability of pollen transfer once the vectors have been attracted to the flower (Fægri & Van der Pijl 1979). Also known as "pollination syndromes", certain floral features have been associated with pollination by certain vectors. For example, birds are primarily attracted by brightly coloured flowers, such as red and orange, while visual cues are less important for nocturnal pollinators (Young 1986). Plants that are thought to be pollinated primarily by non-flying mammals are dully coloured, with a strongly "musky" odour (Carpenter 1978). As well as attractants for pollinators, flower features have evolved to facilitate pollination by certain pollinator groups. For example, in *Banksia*, hooked styles have been associated with pollination by non-flying mammals (Carpenter 1978), while straight styles are thought to be bird pollinated. This has been questioned by Hopper (1980), who demonstrated that pollen from hooked and straight styled species of *Banksia* were found on both types of animal.

Banksia robur inflorescences are a deep metallic green when in bud and are inconspicuous among the dark green foliage, becoming golden yellow when fully open. *B. oblongifolia* inflorescences are similar in colour, but paler: they have a green-grey colour when in bud and are pale yellow when the flowers are fully open. Both these species have straight styles and their inflorescences tend to be in an exposed position on

the plant. Secretion of nectar begins in both species when only a few flowers are open, so the inflorescences at this stage would be visually obscure, but the inflorescences of these species, particularly *B. robur*, have a rather strong sweet, fermented smell. These characters suggest that these species of *Banksia* could potentially attract and use as pollinators birds, mammals and insects.

Although not quantified, visits to inflorescences of all morphs of *Banksia* by insects (including flies, ants and introduced honey-bees) were also observed. However, it is considered that vertebrate foragers have the greater potential to effect pollination. Although insects, such as the introduced honey bee (*Apis mellifera*), were observed foraging on *Banksia* inflorescences, and are found to carry some quantities of pollen, it is generally considered that they are not major pollinators, simply because of their size and foraging behaviour. As bees tend to collect nectar at the base of the flowers (pers obs.), their pollen load would not frequently come into contact with the stigmatic surface, as in the case of another Proteaceous species, *Grevillea barklyana* (Taylor & Whelan 1988). However, it is thought that there is no adverse affect of the foraging of bees on ultimate levels of seed set in some species of *Banksia* (D.C. Paton, pers comm., Vaughton 1992, although see Hill 1982).

The foraging behaviour of visitors to the inflorescences, together with the flowering pattern of the inflorescence is important to their potential as pollinators. Carthew (1991) suggested that birds were less efficient than mammals as pollinators of *Banksia spinulosa*, as they tended to perch on branches adjacent to an inflorescence, and fed only from the advancing front of newly opened flowers. Therefore, they would come in contact with flowers at the male stage only, and not with flowers with receptive stigmas. In contrast, the mammals foraged by crawling all over the inflorescence. However, birds may be less effective in this system because, in *Banksia spinulosa*, flowers open basipetally on the inflorescence. *Banksia robur* and *B. oblongifolia* flowers, as do the flowers of most other species of *Banksia* (George 1981), open acropetally, which

suggests that, even if birds simply perch adjacent to the inflorescence, there is more chance that some part of their body will come in contact with flowers in both male and female phases. The foraging behaviour of the mammals would not change. Therefore the relative importance of each of these types of animal as potential pollinators would come down to the frequency with which they visit the inflorescences.

5.4.2. Birds as potential pollinators

The larger honeyeaters, the Red wattlebird and New Holland honeyeater, were the primary avian visitors observed, both in the formal study presented in this Chapter, and as observed informally in the field. Both these species of bird are aggressive during their attempts to forage, frequently chasing off other individuals of the same and different species (pers. obs.). This may explain the paucity of observed foraging bouts of other less aggressive species of birds, such as the Eastern spinebill, commonly reported as being major visitors of other species of *Banksia*. Eastern spinebills have been reported to visit *B. spinulosa* (Vaughton 1990, Carthew 1991), but were observed in only one foraging bout, on several *B. oblongifolia* plants. Within each foraging bout observed, the wattlebird and honeyeater each visited at least two plants, which suggests that there was a high proportion of interplant pollen movement. While the majority of sequential visits to inflorescences were within the same species (63.5%), a considerable proportion were between species (19.2%) and between a parental and an apparently hybrid individual (17.3%).

There seemed to be a slight preference for foraging in *Banksia robur* inflorescences, as they were visited more often than was expected from the proportion of each morph in flower. This suggests that there may be *B. robur* pollen may be primarily dispersed amongst its own kind, while *B. oblongifolia* and the hybrids within the populations may receive a substantial proportion of *B. robur* pollen. This result reinforces the suggestion in Chapter Four that *B. robur* has less opportunity to pollinate *B. oblongifolia* or the hybrids within the population than *B. oblongifolia* has to pollinate *B. robur*. This result

also supports the notion raised in Chapter Three that the gene flow in the *B. robur/B. oblongifolia* hybrid zone is asymmetrical hybrid zone.

Generally, when more than one inflorescence was visited per foraging bout, the majority were between different plants. Inflorescences visited successively were within fairly short distances of each other. The majority of successive visits were intraspecific. However, because both species were observed to be visited, the large proportion of intraspecific foraging bouts simply suggests that the birds will tend to fly the shortest distance between subsequent inflorescences. Short distances travelled between subsequent stints of foraging is a common conclusion of many other studies. It is generally thought that this pattern of foraging maximises net energy gain (Pyke 1988). This pattern would be predicted for both nectivorous birds and non-flying mammals.

The scale of pollen movement in a system seems to be dependent on the pollen vector the plant utilizes. For example, in some studies, the mean distance flown by insects (bees and wasps) is no different to the pollen flow (Waddington 1981; Peakall 1989). In *Delphinium nelsonii*, pollen flow effected by bird pollination, though again not much different to the mean distance flown by the bird, is markedly different to the pollen flow achieved by the bumble-bee in the same system (Waser 1988). In addition, differences in bird foraging behaviour and habits will have an effect on the extent of gene flow (Linhart *et al.* 1987). It is, therefore, difficult to predict the pollen dispersal from system to system. However, although pollen carry-over was not estimated in this study, Paton (1982) suggests that, based on circumstantial evidence, cross-pollen load of a honeyeater will last through many probes during a visit to an individual plant or inflorescence. So the cross-pollen load of a honeyeater foraging in *Banksia* will generally last longer than the visit to one inflorescence. Therefore, if pollen-carryover is important in the dispersal of pollen in the *Banksia robur/B. oblongifolia* system, interspecific pollen transfer may occur more often than is indicated by the direct observation of the sequence of plants visited by the foraging birds.

5.4.3. Mammals as pollinators

It is difficult to conclude much about the role of non-flying mammals as pollinators of *Banksia robur* and *B. oblongifolia*, and their possible influence on the hybridization of these two species. Only one species of mammal, *Antechinus stuartii* was trapped, which is in contrast to some other studies looking at mammal visitation to other *Banksia* species. Previous studies have shown that an array of non-flying mammals do visit other *Banksia* species. Goldingay *et al.* (1987) trapped pygmy possums, sugar gliders and bush rats, as well as antechinus, within *B. spinulosa*; Turner (1984) found that pygmy possums visited inflorescences of *B. integrifolia*, *B. marginata*, *B. serrata* and *B. spinulosa*. However, in contrast to this study, these studies were conducted in woodlands, in which the species observed were the principally arboreal sugar gliders and pygmy possums.

In addition to the lack of diversity of mammals caught, the actual number of antechinus caught was fairly small. The trapping success of 4.7% in this study is very low compared, for example, to the 42% trap success reported in Carthew (1991). This could suggest that foraging by mammals is comparatively infrequent within these species of *Banksia*. There could be several reasons for this: there may simply be fewer animals present within the swamp, or smaller potential gain for foragers. The number of inflorescences per plant secreting nectar at any one time is fairly low (Chapter Four), so that the gain from foraging would be acquired from a much smaller area within the adjacent woodland.

The animals caught were trapped near inflorescences of all three morphs, suggesting that antechinus will potentially forage in the inflorescences of both species and intermediate types. This was confirmed by the chance direct observation of *Antechinus stuartii* foraging within the inflorescences of the *B. robur* and *B. oblongifolia* mixed stand whilst collecting bird foraging data, between about 6.30 and 8.30 am, on three separate mornings. These mammals are generally nocturnal, although they are known to forage

diurnally if food supply is low (Strahan 1983). On two of the three occasions, a foraging sequence was followed, which included visits to all three morphs. These observed sequences were probably not the complete foraging bout, but they indicate that *Antechinus stuartii* will forage indiscriminantly on these species, and will visit all three morphs within a bout, perhaps contributing to interspecific pollen transfer.

5.4.4. Pollen dispersal and gene flow

The potential for interspecific pollen transfer within the *B. robur/B. oblongifolia* hybrid zone cannot be assessed by tracing the movements of the NSdh s allele, because this allele is restricted to *B. oblongifolia* individuals and plants of hybrid origin. However, this technique enables the comparison of distance travelled by the potential pollen vector and the actual distance dispersed by pollen carrying a marker allele, and is therefore useful as an indication the potential extent of genetic heterogeneity within the population.

5.4.4.1. Assumptions in determining pollen dispersal

In determining the neighbourhood size and area using the NSdh s allele, there is the underlying assumption that this locus is selectively neutral. It was also assumed that the pollen source plants were among those within the populations that were able to be genotyped. This analysis also ignores the potential for longer distance pollen dispersal (i.e. source of pollen may be outside the limits of the hybrid zone), but this is unlikely, as the potential pollinators observed within the hybrid zone forage in a fairly restricted area. The detection of only four variable loci will make it less likely that plants will have a "unique" genotype. There were only a few instances in this study where this problem was encountered: the potential pollen donor plant closest to the maternal plant was used in these circumstances.

5.4.4.2. Patterns of pollen dispersal within the hybrid zone

The leptokurtic pattern of pollen dispersal, commonly observed in plant (Wyatt, 1983, Loveless & Hamrick 1984, Cahalan & Gliddon 1986) and sessile animal (Grosberg

1991) systems, was also apparent in this study. Despite this, the sources of the pollen siring the progeny in a particular plant were not necessarily close neighbours, but the majority of recipients were within about 10 metres. Leptokurtosis in the distance moved by the pollen indicates that the pollen movement, and therefore the gene dispersal, is restricted. This is confirmed by the estimates of effective population size and neighbourhood area using the pollinator flight distances and the movement of the marker allele. Estimates of N_e of little more than one for the marker allele and of only up to 11 for the bird flights indicates that the pollen flow is extremely localized, and differentiation is potentially considerable (Wright 1946).

These estimates of effective population size and neighbourhood area assume that there is no selection against the progeny prior to formation. Although the results of Chapter Three imply that there may be some selection acting against the hybrids in the hybrid zone, the gene flow described in this Chapter is primarily amongst plants of hybrid progeny. Further, the selection against hybrids implied by the results of Chapter Three does not specify the stage at which selection is occurring. If, as may be suggested by evidence, there is a strong association of genotypes to the environment, selection may occur post-dispersal (this is also suggested by the results of the next Chapter). Therefore the progeny array examined in this Chapter will be prior to going through the primary selective process.

This technique does not provide any information on the frequency of pollen between *B. robur* and *B. oblongifolia*, as the marker allele used was not present at all in *B. robur*, nor in a high frequency in *B. oblongifolia*. Tracking the movement of the s allele on the NSdh does provide information on the pattern of dispersal of the whole genome, if it is subject to equivalent selective pressures to all other loci. The small effective neighbourhood sizes obtained in this study suggest that pollen flow is restricted amongst fairly close neighbours, suggesting that there is potentially substantial genetic differentiation. In this population, this may result in the species essentially keeping their genetic integrity, except

genetic integrity, except when they co-occur within the neighbourhood area. If they do occur in close proximity, then gene flow between the species would be subject to flowering synchrony, which is itself limited. Disregarding selection, progression of gene flow across the hybrid zone in this situation would occur extremely slowly.

Estimates of N_e and N_A obtained from the movement of the genetic marker were smaller than those estimated using the observation of bird flights. This result is contrary to many other studies, which have found that the estimation of pollen dispersal through the distance travelled by the potential pollen vector greatly underestimated the distance moved by a marker allele (e.g. Schaal 1980, Handel 1983, Campbell 1991). The discrepancies in these published studies have been attributed to the amount of pollen carryover from one plant to another (Waser & Price 1983, Smyth & Hamrick 1987, Campbell & Waser 1989), and indeed pollen carryover may have influenced the patterns of gene dispersal within the *B. robur/B. oblongifolia* stand. The published studies seem to assume that pollinators transfer out from a pollen source. This is not always the case, as the birds observed in this study will double back within their foraging bout, potentially producing a random spread of pollen, not necessarily radiating from the pollen source plant. Pollen carryover could, in these circumstances, cause gene dispersal to be closer to the source than would be indicated by the flight distances of the birds. Another explanation may be that pollen flow is strictly confined to a particular area because of the possibility of selection when genes are dispersed outside their neighbourhood, a phenomenon that has recently been dubbed "outbreeding depression" (Waser & Price 1983). While reduced fitness of long distance outcrossing has been documented within populations of the one species (e.g. *Espeletia schultzii* , Sobrevila 1988), the concept of outbreeding depression may be particularly relevant when dealing with hybridizing species that are associated with an environmental cline. An allele that is dispersed outside the area/neighbourhood to which it is most suited will have little chance of establishment and survival.

No *B. robur* plants were found to have the NSdh s allele, indicating that this allele is restricted to hybrid and *B. oblongifolia* plants. Indeed, this allele seemed to be largely restricted to the hybrids and *B. oblongifolia* in the mixed stand populations. It is not unusual for unique alleles to arise within hybrid zones (e.g. in *Mus* [Hunt & Selander 1973] and *Rana* [Sage & Selander 1979]), and these have been thought to arise as a result of intragenic recombination (Golding & Stroebeck 1983). This allele was detected in a very small frequency in the pure Darkes Forest *B. oblongifolia* population (see allele frequencies, Table 2.2), but this may have been due to a chance long distance dispersal event. The foraging behaviour of the potential pollen vectors does not suggest this dispersal distance (which would be greater than 2 kilometres) would be common, but may be facilitated as a result of pollen carryover. Pollen carryover may further enable pollen from more than one source to be transferred simultaneously, providing one explanation for multiple fathers in a half-sibling array, although in the case of *Banksia*, sequential opening of flowers, means that the transfer of pollen from different sources can happen over the flowering time of the inflorescence.

Variation in the patterns of pollen dispersal between populations, seasons, and within seasons have been detected in some systems (Campbell & Waser 1989). This variation in pollen dispersal between populations and seasons is because the distance and level of gene flow is primarily dictated by the foraging behaviour of the potential pollinators, which is, in turn, influenced by the flowering time of the plants (Stephenson 1982, Schmitt 1983b, Campbell 1985). Nearest neighbours do not necessarily have synchronous flowering times, and flowering time is not necessarily spatially or temporally consistent. Within the *B. robur*/*B. oblongifolia* system, levels of flowering between populations, between years and among individual plants was remarkably consistent (Chapter Four). Accordingly, the estimates obtained for the effective population size and neighbourhood area obtained through genetic means was fairly similar between populations, indicating that the patterns of pollen dispersal were consistent between populations. Estimates were not calculated over a number of seasons,

nor at intervals throughout the flowering season. However, it may be expected that the effective population size and neighbourhood area may not vary between years, nor even within seasons, although the morph composition of the neighbourhood may vary.

None of gene dispersal variances estimated using the marker allele determined in this Chapter are comparable to the dispersal variances in the centre of the cline, determined in Chapter Three. However, the estimate of σ^2 from the cline shape of the the two populations are similar to (as in the case of the Cataract population), or much larger than (Darkes Forest population) the distances flown by the birds between successive inflorescences. This suggests two things about the system. Firstly that pollen carryover may be an important factor in this system, but the carryover distance may not be as far as the distance flown in a foraging bout (particularly in the Cataract population). Secondly and further, the s allele on NSdh may not be selectively neutral, and so the neighbourhood size calculated using these alleles may be smaller than those obtained using the other loci which determined the cline shape dispersal variances. Large dispersal distances within the centre of the cline will require greater selection to override its effect to maintain the cline (Endler 1977, Szymura & Barton 1986), and this selection may have a great effect on this locus.

The differential selection occurring on the system is difficult to detect when only the pre-mating or post-zygotic stages are available for study. Selection may occur at the post-mating stage, creating the observed gap between the bird foraging patterns and the gene dispersal using the NSdh. A study of this stage of development will be made in the following chapter, through the use of controlled pollination experiments. The effect of neighbourhood size and pollen flow restriction suggested in this chapter on the mating system of the species and the interspecific gene flow will also be addressed in Chapter Six.

Chapter Six

Mating system and levels of hybridization in *Banksia robur* and *Banksia oblongifolia.*

6.1 Introduction

The previous two Chapters showed that there is only limited pre-pollination reproductive isolation between *Banksia robur* and *Banksia oblongifolia.* Some synchrony of flowering time and the interspecific foraging of the potential pollinators provide the possibility for gene exchange between these two species. However, the resultant proportion of progeny that are hybrids, after the deposition of unlike pollen on a stigma, is largely dependent on processes controlled by the maternal plant.

6.1.1. Selection against incompatible pollen

The study of pollination within many plant species has suggested that there are many stages after pollen deposition at which selection against a particular genotype can occur. These are usually divided into either pre-zygotic or post-zygotic. Pre-zygotic processes occur at the level of the stigma, upper or lower style and the ovule, while post-zygotic occur at the different stages of seed development. Pollen can "compete" by exhibiting allelopathic effects on other pollen types (Thompson *et al.* 1981) or through differential rates of pollen germination and tube growth (Snow & Mazer 1988). Often these pre-zygotic selection processes are thought to be controlled at least partially by the maternal parent (Marshall 1988). Post-zygotic selection usually acts through the non-random abortion of zygotes (Pham & Bougerol 1993).

Like incompatible intraspecific pollinations, interspecific pollinations may also be selected against at the post-pollination stage, because even when natural hybridization is known to occur, low seed set is often found in controlled interspecific hand-pollinations (Borgen

1976, Bernhardt & Calder 1981). Drake (1981a) outlined a model for *Eucalyptus*, indicating the stages at which possible loss of a hybrid seed could occur, suggesting that the pollen of one species deposited on the stigma of another is subject to the same selective screening as if it was deposited on the stigma of a con-specific plant. Indeed, in *Eucalyptus*, while interspecific pollen often readily germinates on the stigma, early inhibition has been found to occur in the upper style, through the abnormal growth of pollen tubes (Ellis *et al.* 1991).

6.1.2. *Banksia* breeding system and the potential for hybridization

Most studies on the breeding systems in the genus *Banksia* suggest that they are preferential outcrossers, and that they set seed by self-fertilization only infrequently (Scott 1980, Lewis & Bell 1981, Carthew *et al.* 1988, Vaughton 1988, Carthew 1991, Goldingay *et al.* 1991, Coates & Sokolowski 1992). Carthew (1991) concluded that in *B. spinulosa* var. *spinulosa*, preferential outcrossing was the result of a maternal mate choice at a late pre-zygotic stage, because at the stage of early pollen germination and tube growth, there was essentially no difference between outcross and self pollen. Likewise, Lewis and Bell (1981) showed that pollen from some species of *Banksia* will readily germinate on the stigmas of quite a wide range of other species, indicating that incompatible pollen is not screened at this early stage. There are no published records of the observation of the progress of interspecific pollinations in *Banksia* beyond the stigmatic region. However, the growth of self pollen within a style may provide some insight in the stages of selection against incompatible pollen within *Banksia* (Fuss & Sedgley 1991a & b). In contrast to the conclusions of Carthew (1991), Fuss and Sedgley (1991a) found that selection against self pollen in *Banksia coccinea* occurred at the early pre-zygotic stage, through malformation of the pollen tube in the upper style, similar to those mutations found in *Eucalyptus* (Ellis *et al.* 1991). Once the pollen tube has grown beyond the upper style region, the next stage at which selection against incompatible pollination is likely to occur in *Banksia* is post-zygotically, through the selective abortion of immature seeds (Fuss & Sedgley 1991a).

6.1.3. Plant mating systems

The mating system a population exhibits is the culmination of the processes of pollination outlined above. Patterns of inheritance are commonly inferred from indirect determination of gene flow, via observations of flowering time and pollinators, floral morphology and breeding experiments (e.g. Schmitt 1983b, Webb & Bawa 1983). These methods are useful in estimates of the patterns of inheritance in the progeny, but can only provide qualitative predictions of the gene flow and mating system of a group of plants (Jain 1978, Clegg 1980). A more direct approach is the comparison of the proportion of progeny that were produced as a result of self fertilization and those produced as a result of pollination by another plant using genetic markers, such as allozymes.

Following pollination and fertilization, the types of successful matings (i.e. selfing, intraspecific outcross or interspecific cross) will be evident in the genotypes of the progeny array from the plants in the population. Two parameters, the outcrossing rate and Wright's Fixation Index, are commonly used to describe a plant population's mating system. The first is the outcrossing rate (t), which simply compares the genotypes of the plants to that of their progeny, providing an estimate of the proportion of seeds produced as a result of pollination by genetically unlike plants. t will equal one in totally outcrossing systems. The second parameter, Wright's Fixation Index (F) (the coefficient of inbreeding), indicates heterozygote deficiencies within the progeny which are inferred to be a result of mating between closely related individuals. F approaches unity if the progeny are inbred, and zero in the case of panmixis. The accuracy of these parameters in describing the mating system of the population is subject to the assumptions of the mixed mating model, which state that the alleles within the population must segregate in Mendelian ratios, be in linkage equilibrium and the pollen must be randomly dispersed within the population (Brown *et al.* 1975, Clegg 1980, Ritland & Jain 1981). These assumptions need to be addressed within the populations for which the mating system is being determined.

The level of outcrossing versus selfing within a species is linked to the proportion of pollen types within the pollen pool at a given time. The pollen pool associated with two sympatric species will also affect the frequency of hybrid seed produced in the population. In a pollen environment where interspecific pollen is more plentiful than intraspecific pollen, the only real options for seed production are selfing and hybridization. In a predominantly outcrossing species, intraspecific pollen may be more prepotent than self pollen, making hybrid production almost inevitable when pollen from another species predominates. This situation has been described in *Iris* (Cruzan *et al* 1994), where hybrids were more likely to be produced by a species when the other species' pollen was more plentiful in the pollen pool.

6.1.4. Natural levels of hybridization

While the outcrossing rate describes the proportion of seeds produced as a result of selfing and outcrossing within a population of the one species, the level of hybridization can be assessed simply through the determination of the genotypes of the seeds produced by the plants within a mixed species stand. Determination of seed genotype will only indicate the proportion of hybrids produced, while the actual proportion that will establish and grow in future populations is initially dependent upon their germination success. Many studies have examined germination of seed under laboratory conditions, but these may overestimate the establishment success of the seeds, because of the absence of the selection processes to which the seed is usually subjected (such as environmental unsuitability, competition with other seeds and exposure to seed eaters) under field conditions (Harper 1977). In monospecific stands, the genetic differences between seedlings may contribute to differential fitness in their establishment (Schmitt & Antonovics 1986), and this may be extended to hybrid populations, where genetically hybrid seed may germinate and establish in regions to which the parental genotypes are not suited and vice versa. However, field experiments may underestimate the success of the types of seed because they are placed in locations to which they may not be naturally

dispersed. In order to determine the propensity for germination of hybrid and parental seed, germination trials in the laboratory and under field conditions should be undertaken.

This Chapter describes the mating systems of *B. oblongifolia*, and determines the proportion of hybrid production between the two species the levels at which selection against hybrid progeny may be occurring. A hand pollination experiment was conducted to assess:

(i) the potential for selfing versus outcrossing within *B. robur* and *B.oblongifolia*,

(ii) the potential for hybridization between these two species, and

(iii) gametic segregation patterns in the two species, by the determination of any detectable deviation of the resultant genotypes from Mendelian inheritance ratios amongst the progeny.

The results of the breeding experiment were compared to the genotypes of naturally produced progeny within the hybrid zones. This analysis allowed the determination of:

(iv) the mating system of *B. oblongifolia* within the pure stand populations determination of the outcrossing rate, Wright's fixation index and the inbreeding coefficient expected under inbreeding equilibrium, in order to assess the selfing versus outcrossing potential within the species. The mating system for pure stand *B. robur* was not determined because this species was genetically invariant.

Analysis of the genotypes of the progeny in an array of maternal plants allowed the assessment of:

(v) hybridization within the mixed stands through the determination of the proportion of hybrid seed produced for each *B. robur* and *B. oblongifolia* plan

(vi) the relative proportions of F_2 hybrids and backcrossed progeny, determined by the progeny of putative F_1 hybrid maternal plants and the maternal plants assessed as F_2 hybrids or backcrosses.

(vii) the relative proportions of hybrids versus parental progeny compared to the expectations generated from (a) the proportion of mature hybrid origin parental plants established within the populations, (b) the potential proportion of hybrids and parental seeds estimated from a combination of random mating within the hybrid zones and the synchrony of inflorescence flowering, determined in Chapter Four and (c) the proportion of seed with GHIS of 3 produced by parental plants expected from random mating within the hybrid zones.

Finally, a survey of the germination of a range of genotypes under laboratory and field conditions were used to determine:

(viii) the ability of a range of parental and hybrid genotypes to germinate, and

(ix) the ability for parental and hybrid seeds to germinate under field conditions, in a post-fire environment.

These trials were performed to indicate if post-dispersal selection may be acting in this system.

6.2. Methods

6.2.1. Pollination experiment

6.2.1.1. Experimental Design

Hand pollination experiments were conducted during the flowering season of 1990, between February and July, in each of the Cataract and Darkes Forest populations.

In each population, 30 plants of each species from pure stand regions (see Chapter One) were chosen, to ensure that the plants were parental species, and not of hybrid origin. The plants selected had at least one inflorescence with no flowers yet open. All other

inflorescences on the plant were removed (see Goldingay *et al.* 1991), to reduce the possibility that outcomes would be confounded by resource partitioning among inflorescences within a plant (Wallace & O'Dowd 1989). The plants were randomly allocated to one of three treatments, so that each treatment had ten replicates in each species in each population:

(i) Self - pollen was removed from newly opened flowers on the inflorescence to pollinate receptive flowers on the same inflorescence;

(ii) Intraspecific cross - flowers were pollinated with pollen from other plants within the experiment of the same species;

(iii) Interspecific cross - inflorescences were pollinated with pollen from plants of the other species within the experiment. The pollen used had been removed for other treatments.

The pollen from only one plant was used to pollinate each inflorescence.

In order to prevent visits from possible pollinators, each inflorescence was enclosed in a cage of plastic gutter guard, covered in 1mm fly mesh, before any flowers had opened. However, the fly screen and plastic gutter guard of some of the cages were occasionally chewed through by small mammals (see Whelan and Goldingay 1986). These inflorescences were excluded from the experiment, and were replaced by other inflorescences fitting the original criteria.

Pollen was removed from opened flowers using a small square of clean flannel. The use of a cloth made it easier to control the possibility of contamination of one treatment with inappropriate pollen types, than if uncovered fingers were used. The flowers with newly cleaned stigmas were then left for two days before treatment (which is about the time after pollen removal when the stigma becomes fully receptive), when the appropriate pollen was then applied to the style tips. For the self-pollination treatment, pollen was removed from newly open flowers, and then applied to the styles on the same inflorescence that had had pollen removed two days previously. For both the cross-pollination treatments,

pollen was removed from the flowers, and this freshly removed pollen was then used to pollinate stigmas of another inflorescence ready for pollination. Treatments were carried out every two days until all the flowers were open and had been treated. This resulted in about 4 or 5 applications of pollen per inflorescence.

6.2.1.2. Pollen Tube Growth

To determine the effectiveness of hand pollinations in the transfer of pollen and to assess the levels of pre-zygotic pollination success of each treatment, pollen tube growth within a sample of styles from each manipulated inflorescence was assessed. One week after treatment was completed, 10 styles from each inflorescence were collected. The styles were fixed in a 3:1 solution of 100% ethanol: glacial acetic acid for a minimum of one night, then cleared in 1M NaOH for a minimum of 2 days, placed in a 10% decolourised analine blue solution and observed using fluorescence microscopy (Martin 1959).

Two analyses were carried out on the resultant pollen tube data. Firstly, the proportion of styles from each treated inflorescence was transformed using the arcsine transformation, to normalize the data (Zar 1984). The second data set consisted of the number of pollen tubes per style with pollen tubes. Both these sets of data were analysed using a Model 1 Three Factor ANOVA (with treatment, species and population all being fixed factors), to determine if there were significant differences between treatments, species and populations in the occurrence and level of pollen tube growth.

Infection of the transmitting tissue by fungus was observed in most styles. To determine if fungal growth was associated with the treatment, the proportion of styles with fungal growth was transformed using an arcsine transformation. These transformed data were then analysed in a Model 1 Three Factor ANOVA (with factors again being treatment, species and population) (Zar 1984).

6.2.1.3. Seed set

Eighteen months after the pollinations were completed, the number of inflorescences that set seed and the number of follicles on each infructescence were counted. These infructescences were collected so the seeds could be genotyped using gel electrophoresis.

Seeds were extracted from the infructescence as described in Chapter Two. Seeds that were underdeveloped (seed shrivelled) or infected by fungus were tallied, but not used in the genetic determinations. The healthy, fully matured seeds were placed on wet filter paper to imbibe overnight. The seed coat was removed and the seed ground for electrophoresis, according to methods presented in Chapter Two. Gels were stained for three enzymes yielding four loci: *Adh*, NSdh, *Sod* and *Gdh*.

6.2.2. Mating system of *B. oblongifolia*

The mating system was determined for *B. oblongifolia* in the pure populations. For each locus, the two parameters, the outcrossing rate (t) and Wright's Fixation Index (F), were calculated to determine the breeding system in all groups of plants surveyed. Single-locus estimates of t and F were calculated using the methods of Brown *et al.* (1975), while a multi-locus estimate of outcrossing was also calculated (Ritland & Jain 1981). The multi-locus estimate of t is considered to more accurately determine the level of outcrossing, since it considers all the genotypes of the progeny at all loci, and is more robust under violations of the mixed-model mating system (Ritland 1983). The multi-locus estimate of t was used to estimate the inbreeding coefficient expected under inbreeding equilibrium (F_e), using the expression:

$$F_e = (1 - t)(1 + t).$$

This parameter indicates the level of self fertilization that would be expected given the calculated t value.

The calculation of t and F assume that: (i) the alleles segregate according to Mendelian ratios, (ii) pollen is randomly dispersed within the population, and (iii) the loci are inherited independently of one another. The first assumption was addressed using the hand-pollination experiment, while the last two were determined using the genotype frequencies within the progeny arrays.

6.2.3. Assumptions of the mixed mating model

Differences among plants in the estimated pollen gene frequencies were assessed using chi-squared tests of heterogeneity (Brown *et al.* 1975). At each locus, the number of heterozygous (the detectable outcrosses) and homozygous progeny were compared for each homozygous maternal plant, using contingency table analysis.

Valid estimation of both single-locus and multi-locus outcrossing rates require that loci are inherited independently of each other, i.e. they are in linkage equilibrium (Brown *et al.* 1975, Ritland & Jain 1981). To determine if there were significant associations between loci, Burrow's composite measure of linkage disequilibrium (Δ_{ij}) was calculated (Brown & Weir 1983 [with corrections - S. Carthew pers. comm.]), using only the *B. oblongifolia* populations. This parameter indicates the deviation due to non-random association of multi-locus genotypes. It was used in preference to Hill's measure of gametic disequilibrium, used in Chapter Three, as Hill's measure assumes the random union of gametes, an assumption that could not be verified for the data in this portion of the study (Brown & Weir 1983).

6.2.4. Hybrid seed production

6.2.4.1. Genotypic makeup of hybrid zone progeny

Seeds from the two populations were collected and genotypes determined as described in Chapter Two. The three-locus genotype of the progeny was determined and each seed was given a score on the genetic hybrid index (GHIS) (Chapter Two). The proportion of progeny with each hybrid index score was determined for the parental plants within the

mixed stands (i.e. those plants scoring either 0 [*B.robur*] or 6 [*B. oblongifolia*]). In each case, those progeny scoring 3 were confidently classified as F_1 hybrids (i.e. they were exactly intermediate between the two parental genotypes), while those scoring 0 or 6 were considered to result from intraspecific pollinations. Progeny with GHIS's of 1 and 2 from *B. robur* maternal plants, and with GHIS's of 4 or 5 from *B. oblongifolia* plants were classified as backcrosses.

The same procedures of classifying seeds were used for maternal plants of hybrid origin. The maternal plants within the mixed stands with GHIS of 3 were considered to be F_1 hybrids, so their progeny were considered to be F_2 hybrids or backcrosses. The GHIS's of the progeny would be expected to extend along the whole range the genetic hybrid index. The proportion of seed with each GHIS in the later generations was determined from the remaining maternal plants within the mixed stands, which were a mixture of F_2, backcrossed and later generation adult plants.

6.2.4.2. Expectations of progeny types

Three sets of expectations predicting the makeup of the progeny within the hybrid zones were used based on information in previous chapters.

The first set of expectations was based on a direct comparison between the proportion of each genotype (simplified through the use of genetic hybrid index) of the plants established within the hybrid zones (calculated in Chapter Two) and the proportion of each GHIS amongst the seed produced by the plants. Significant heterogeneity amongst expected and observed GHIS's were tested using a χ^2 goodness of fit test.

The second set of expectations was the potential proportion of fruits produced by an inflorescence through pollination by intraspecific, interspecific and hybrid pollen, derived from the proportion of pollen of each type available within the pollen pool at a given time (Chapter Four). In this test only *B. robur* and *B. oblongifolia* plants were used because it

was impossible to divide the progeny of the hybrid plants into those produced through pollination by *B. robur*, *B. oblongifolia* and hybrid pollen. Again, a χ^2 goodness of fit test was used to determine significant heterogeneity amongst expected and observed numbers of progeny.

The last set of expectations were derived from a combination of the proportion of plants with each GHIS within the hybrid zone, the flowering synchrony of each GHIS and the proportion of each GHIS within the pollen pool at the times of flowering synchrony. For simplicity, the data set was narrowed to the predicted and actual number of seed with a GHIS of 3 produced from parental plants (plants with GHISs of 0 and 6), although it must be emphasized that the seed with GHIS of 3 were not necessarily F_1 hybrids. A χ^2 goodness of fit test was used to determine significant heterogeneity between expected and observed numbers of seed with GHIS of 3 produced by the parental plants.

6.2.5. *In vitro* seed germination

The germination success was determined for seed from an array of plants within the hybrid zone in the Darkes Forest population. All seeds were imbibed overnight in preparation for electrophoresis. Each seed was then cut in half: the half containing the embryo was placed on water soaked cartridge or tissue paper and left to germinate, while the other half of the seed was assayed to determine its genotype. Successful germination was concluded with the emergence of the root radical.

6.2.6. Transplant Experiment

The experiment was conducted in a recently burned site within the Water Board Catchment (150°55'37"E, 34°15'44"S see Figure 1.1). The site was burnt in November 1990, and seed were planted in December 1990. It was at this time that seeds were being dispersed from the bradysporous fruits of *Banksia* in the area.

Twenty infructescences from pure stand *Banksia robur* and *B. oblongifolia*, and hybrid individuals from a hybrid zone (60 infructescences in total) were collected and the seed extracted. Three hundred seed from the total seed pool of each type were used. The burned site was divided into three natural zones: an area of only *B. robur*, a region consisting of a mixed stand of *B. robur*, *B. oblongifolia* and hybrid plants, and an area consisting primarily of *B. oblongifolia*. Within each of these regions, 10 plants were randomly chosen. Three seed were planted along 10 radii equally spaced around the plant, in the same sequence: along the radius pointing due west, a *B. robur* seed was planted about 30 cm from the plant, a hybrid origin seed, 60 cm from the plant and then a *B. oblongifolia* seed a further 30 cm from the plant; along the next radius, the seed sequence was hybrid, *B. oblongifolia*, then *B. robur*, and so on until 10 seeds of each type were planted. The site where each seed was planted was marked with a labelled stick

The seeds were left for approximately a year, when they were checked for establishment.

6.3. Results

6.3.1. Pollen tube growth

Pollen tube growth was low in all treatments (percentage of styles with tubes range between 2.1 and 8%) (Table 6.1). However, there was no significant heterogeneity in either the number of pollen tubes with styles between treatments, species or populations (Table 6.2a), or in the number of pollen tubes per style with pollen tubes between treatments or species (Table 6.2b).

The transmitting tissue of 75% of all styles observed using fluorescence microscopy had been colonized by fungus (Table 6.1). There was no significant heterogeneity between populations, species and treatment in the proportion of styles with fungal growth (Table 6.3). Although the bright fluorescence of the hyphae may have also masked the detection of pollen tubes in styles where no pollen tubes were seen, there was no conclusive evidence that fungal growth prevented the growth of pollen tubes, since 25% of the styles

Table 6.1. Results of the pollination experiment. The percentage of styles with fungal growth in the transmission tissue, the percentage of styles with pollen tubes, the number of tubes per style with pollen tubes (± standard error), the number of infructescences and the number of follicles per infructescence (figures in brackets are the follicle number on each infructescence) is presented for the three treatments for each species of *Banksia* (the populations were pooled). For each treatment for each species, n=20. The experiment was performed on the two parental species in the two populations described in Chapter 1, but the results are pooled in this table. Three treatments were used: self, intraspecific cross and interspecific cross

Species	Treatment	Percent styles with fungus	Percent styles with tubes	Tubes per style	Number infructescences	Follicles per infructescence
	self	81.5	2.1	1±0	0	0
B. robur	**intra**	78.0	2.1	1.0±0	1	10
	inter	79.2	5.9	1.5±0.1	0	0
	self	78.3	2.2	1±0	1	15
B. oblongifolia	**intra**	72.3	8.0	1.8±0.2	2	44 & 8
	inter	67.3	3.0	1.5±0.2	0	0

Table 6.2. Three-factor ANOVA comparing the effects of population, species and pollination treatment on a. the proportion of styles with pollen tubes and b. the number of pollen tubes per style for styles with tubes. The data in the first ANOVA were arcsine transformed to normalise (Zar 1984). Levels within factors were the populations (Darkes Forest and Cataract), the species (*B. robur* and *B. oblongifolia*) and the three treatments used in the experiment (intraspecific cross, interspecific cross and self). NS indicates that there was no significant heterogeneity detected within each source of variation.

Source of Variation	D.F.	M.S.	F	P
a. Proportion of styles with pollen tubes				
Population	1	0.091	2.622	NS
Species	1	0.004	0.110	NS
Population X Species	1	0.007	0.189	NS
Treatment	2	0.018	0.521	NS
Treatment X Population	2	0.077	2.201	NS
Treatment X Species	2	0.040	1.150	NS
Treat. X Pop. X Sp.	2	0.083	2.377	NS
Error	90	0.035		
b. Pollen tubes per style with tubes				
Population	1	0.462	1.143	NS
Species	1	0.057	0.142	NS
Population X Species	1	0.826	2.045	NS
Treatment	2	0.169	0.418	NS
Treatment X Population	2	0.703	1.741	NS
Treatment X Species	2	0.674	1.668	NS
Treat. X Pop. X Sp.	2	1.029	2.547	NS
Error	89	0.404		

Table 6.3. Three-factor ANOVA comparing the proportion of styles with fungal growth in the transmission tissue. The data were arcsine transformed to normalise. NS indicates that there was no significant heterogeneity detected within each source of variation. The factors are the same as those in Table 6.2.

Source of Variation	D.F.	M.S.	F	P
Population	1	0.230	1.499	NS
Species	1	0.421	2.750	NS
Population X Species	1	0.001	0.005	NS
Treatment	2	0.100	0.650	NS
Treatment X Population	2	0.009	0.061	NS
Treatment X Species	2	0.114	0.743	NS
Treat. X Pop. X Sp.	2	0.288	1.881	NS
Error	90	0.153		

in which there was some pollen tube growth were also infected with fungus. On the other hand, the presence of pollen tubes in infected styles may suggest that pollen tube growth occurred prior to the colonization of transmission tissue of the fungus.

6.3.2. Seed Set

Seed set was low among the treated inflorescences. Only four (3.3%) of the 120 inflorescences treated formed follicles (Table 6.1). The only *B. robur* inflorescence to set seed was an intraspecific cross, while three *B. oblongifolia* inflorescences treated (one selfed and two intraspecifically crossed) set seed. No interspecifically crossed inflorescences of either species set seed.

6.3.3. Genetic confirmation of Mendelian inheritance

The seeds from the sole infructescence from the selfing treatment (a *B. oblongifolia* inflorescence) displayed genotypic ratios expected for self-fertilization with normal Mendelian inheritance (Table 6.4). All of the seed displayed only the parental alleles. This was true even for *Sod* and *Gdh*, where alternate alleles are present in the population at high frequencies. However, the deviations of the genotypic ratios from those expected under Mendelian inheritance could not be tested statistically because of the small sample size.

Similarly, the alleles of the seeds from the two infructescences formed from *B. oblongifolia* intraspecific crosses exhibited only the alleles present in the parental plants, indicating that the hand-pollinations were successful. There were deviations from the ratios expected under Mendelian inheritance when there was more than one allele involved in the cross at that locus in both cases (Table 6.4). A Chi-square goodness of fit, performed on Cross 1, showed that the deviations were significant (*Adh*: $\chi^2=9.1$, $p<0.005$; NSdh: $\chi^2=15.36$, $p<0.001$; *Sod*: $\chi^2=8.5$, $p<0.025$; *Gdh*: $\chi^2=18.96$, $p<0.001$). For cross 2, none of the genotypic ratios were significantly different from

Table 6.4. The four-locus genotypes of the progeny produced by the hand-pollination experiment. Four infructescences were produced from 120 inflorescences produced. The first genotype in the "Parents" column is the seed plant genotype, the other is the pollen genotype. The numbers are the number of seed of that genotype, and the bracketted number is the expected number of seed of that genotype under Mendelian inheritance. * after the parent cross denotes significant deviations from ratios of progeny genotypes expected under Mendelian inheritance.

			Genotype					
	Locus	Parents	AA	AB	BB	AC	BC	CC
B. oblongifolia Self	Adh	BB			4 (4)			
	NSdh	AA	4 (4)					
	Sod	AB	3 (1)	1 (2)	0 (1)			
	Gdh	BC	1 (1)	2 (2)	1 (1)			
B. oblongifolia Intra #1	Adh	BBxAB*		12 (22)	32 (22)			
	NSdh	AAxAB*	35 (22)	9 (22)				
	Sod	ABxAB*	3 (11)	25 (22)	16 (11)			
	Gdh	BCxBC*			23 (11)		21 (11)	3 (11)
B. oblongifolia Intra #2	Adh	BBxBB			8 (8)			
	NSdh	AAxAA	8 (8)					
	Sod	CCxBC					6 (4)	2 (4)
	Gdh	BBxAB		2 (4)	6 (4)			
B robur Intra	Adh	AAxAA	9 (9)					
	NSdh	AAxAA	9 (9)					
	Sod	CCxCC						9 (9)
	Gdh	AAxAA	9 (9)					

those expected under Mendelian inheritance (*Adh* and NSdh: could not be tested statistically; *Sod*: χ^2=2, 0.25>p>0.10; *Gdh*: χ^2=2, 0.25>p>0.10)

The seeds obtained from the *B. robur* outcross replicate contained only the *B robur* alleles, but could not be statistically tested as both the mother and father plants were of the same genotype.

6.3.4. Mating System of *B. oblongifolia*

6.3.4.1. Outcrossing rates

Both the single-locus and multi-locus estimates of outcrossing rate for *B. oblongifolia* were generally high (Table 6.5). Outcrossing rates were high in the populations on the *Sod* and *Adh* loci, while the only estimate available for *Gdh* was low. The mean over the three loci for all populations indicated fairly high rates of outcrossing, although the interpretation of the mean of the single-locus outcrossing rates needs to be made cautiously, since the estimates for both populations were the mean of only two loci. Only the mean of the single-locus estimates in the Darkes Forest population was significantly different to one. The multi-locus estimates of t were also high, and were significantly different to one in both populations.

6.3.4.2. Wright's fixation index

Estimates of the inbreeding coefficient for *B. oblongifolia* were in agreement with the outcrossing rate estimates (Table 6.6). In the Cataract population, the F value of the *Sod* locus was significantly different to 0, as was the mean of the single locus estimates. In the Darkes Forest population, there was no deviation from zero of either single-locus or mean estimates of F. There were, however, large differences between the mean of Wright's Fixation Index over loci, and the inbreeding coefficient expected under inbreeding equilibrium (F_e) in both populations (Table 6.6). Both estimates of F_e were substantially larger than the mean F over the three loci, indicating that the levels of inbreeding are greater than those expected from the multi-locus outcrossing rates.

Table 6.5. Estimates of outcrossing rates of two pure populations of *Banksia oblongifolia*: The table lists outcrossing rate estimates for each locus (single locus estimates [Brown *et al.* 1975]), a mean of the single locus estimates and a multi-locus estimate (determined using the methods of Ritland and Jain 1981). Figures in the brackets are standard errors. * indicates significant departures of t from 1 ($p<0.05$). - indicates that an estimate of the outcrossing rate could not be determined.

Population	Single locus estimates			Mean	Multi-locus
	Adh	Sod	Gdh		
Cataract	-	1.299 (±0.199)	0.203* (±0.278)	0.751 (±0.171)	0.896* (±0.050)
Darkes Forest	0.893 (±0.200)	0.741* (±0.087)	-	0.817* (±0.075)	0.880* (±0.042)

Table 6.6. Estimates of Wright's Fixation Index for two pure populations of *Banksia oblongifolia*. Estimates of F were determined for each locus individually, and a mean over the three loci is also given. Standard errors are given in brackets. * indicates a significant departure of F from 0 ($p<0.05$). The inbreeding coefficient expected under inbreeding equilibrium (F_e) was determined from the relationship $F_e = (1-t)(1+t)$.

Population	Adh	Sod	Gdh	Mean	F_e
Cataract	-	0.236* (±0.065)	0.060 (±0.072)	0.148* (±0.049)	0.436
Darkes Forest	0.058 (±0.285)	0.041 (±0.055)	-	0.050 (±0.145)	0.250

6.3.5. Local heterogeneity of allele frequencies

Significant heterogeneity between plants in pollen allele frequency was detected at all loci tested for both pure populations of *B. oblongifolia* (Table 6.7). Two of the three not significant values of χ^2 (*Sod* Cataract hybrid zone and *Gdh* Darkes Forest hybrid zone) were calculated from only two homozygous plants, which is too small a sample size to detect heterogeneity in the gene frequency of the pollen. For *B. robur*, five of the six tested cases showed significant heterogeneity in pollen allele frequencies (Table 6.7) Estimates of allele frequency heterogeneity for hybrid individuals were not calculated, because there were too few homozygous individuals for a valid statistical test.

6.3.6. Linkage disequilibrium

Tests for linkage between loci, calculated for only *B. oblongifolia*, implied that some pairs of loci did not segregate independently (Table 6.8). Of a total of eight valid tests, five indicated significant deviations from linkage equilibrium. The two estimates of Δ_{ij} (one from each population) showed significant deviations from linkage equilibrium, but were both negative values, suggesting that some genotype combinations were less frequent than would be expected from random association. Within the hybrid zone populations, two combinations from the Cataract population and one from the Darkes Forest population tested for associations showed significant deviations from linkage equilibrium.

6.3.7. Hybrid production

The majority of the seeds produced by infructescences of the parental plants (with GHIS of 0 and 6) had the same genotype as the maternal plant (Figure 6.1a & b). In the Darkes Forest population, 97.5% of the seeds produced by *B. robur* plants (GHIS of 0) had a GHIS of 0, while 65.5% of the seed produced by *B. oblongifolia* plants (GHIS of 6) also had a GHIS of 6. Similarly, in the Cataract population, plants produced a majority of progeny with the same GHIS as the mother: 96.5% of the seed produced by the Cataract

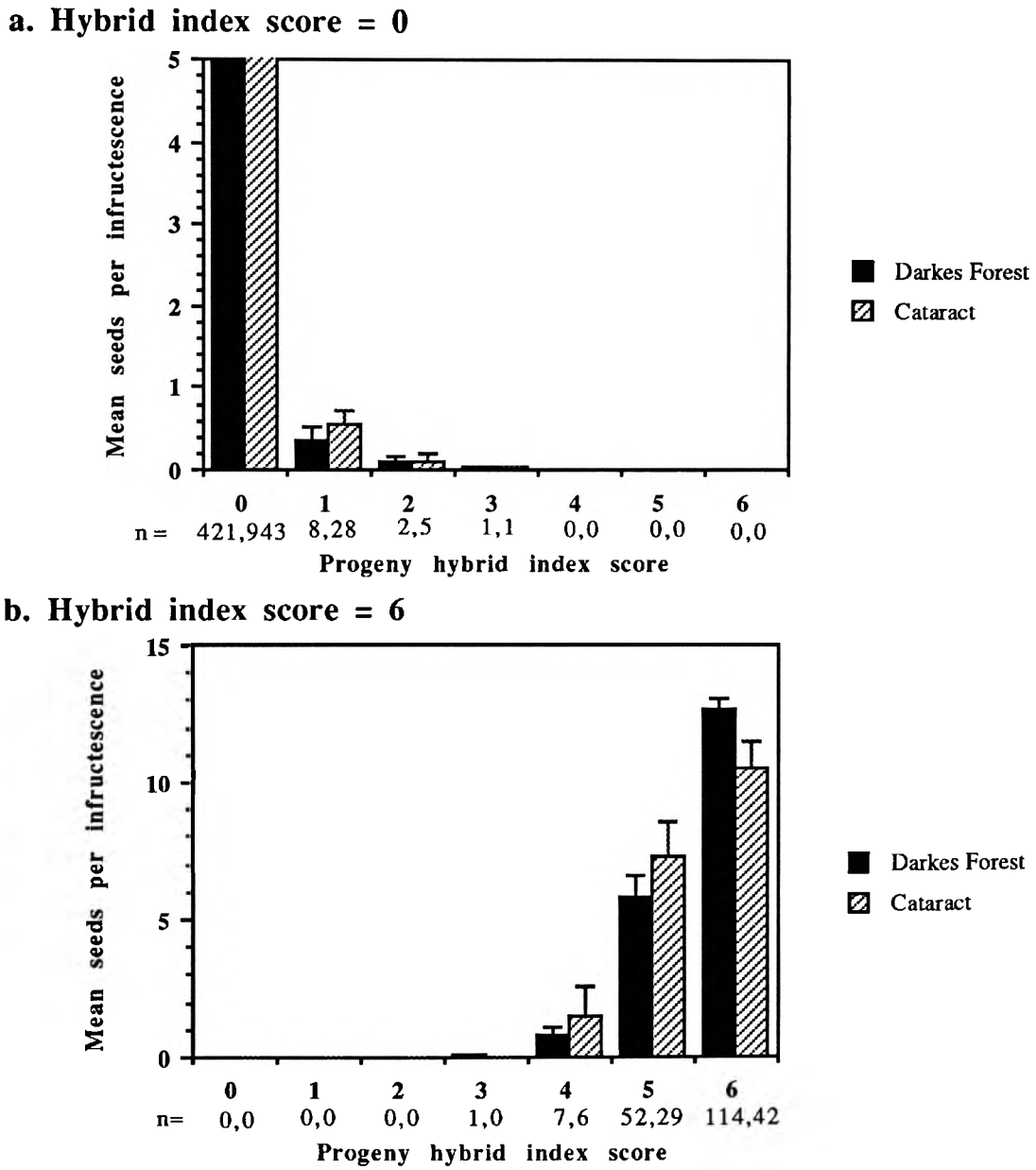
Table 6.7. Chi-square tests of heterogeneity at each locus, for two pure populations of *Banksia oblongifolia*, and two hybrid zone populations each of *B. oblongifolia* and *B. robur*. Asterisks indicate significant heterogeneity detected: * indicates p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001. Yates correction for continuity applied within the calculation indicated by †. No significant heterogeneity indicated by NS. - indicates that there were fewer than two plants within the category homozygote.

Population	Adh	Sod	Gdh
Pure Stand Populations			
Cataract	27.79 (13)**	-	-
Darkes Forest	28.58 (15)*	17.76 (4)***	-
Hybrid Zone Populations			
Banksia oblongifolia			
Cataract	23.47 (9)**	0.156† (1)^{NS}	-
Darkes Forest	24.08 (16)^{NS}	28.24 (6)****	0.119† (1)^{NS}
Banksia robur			
Cataract	104.24 (43)****	134.15 (43)****	90.12 (42)****
Darkes Forest	40.32(13)****	32.75 (13)**	21.05 (13)^{NS}

Table 6.8. Estimates of linkage disequilibrium using Burrow's composite measure (Δ_{ij}) for *B. oblongifolia*. Δ_{ij} were determined between the three variable loci within the pure and hybrid zone populations of *B. oblongifolia*. Standard errors are shown in brackets. * indicates significant deviations of Δ_{ij} from 0 at $\alpha = 0.05$. ND indicates that Δ_{ij} was not determined for that pair of loci (*Adh* in Cataract and *Gdh* in Darkes Forest were invariant).

Population	Δ_{ij} *Adh & Sod*	*Adh & Gdh*	*Sod & Gdh*
Pure Stand			
Cataract	ND	ND	-0.410* (±0.026)
Darkes Forest	-0.041* (±0.009)	ND	ND
Hybrid Zone			
Cataract	-0.0029 (±0.060)	0.158* (±0.018)	0.238* (±0.017)
Darkes Forest	0.004 (±0.007)	0.065* (±0.008)	0.002 (±0.012)

Figure 6.1. The GHISs of the progeny produced by the parental species in each hybrid zone. The mean number of seeds (with standard error bars) produced per infructescence with each score on the genetic hybrid index for a. infructescences from plants with GHIS of 0 (*B. robur*) and b. with GHIS of 6 (*B. oblongifolia*). The total number of seed with each GHIS is shown under the abscissa.



population plants with a GHIS of 0 had a hybrid index score of 0, while the plants with a GHIS of 6 had 54.6% of the progeny with a GHIS of 6.

Seeds with a GHIS of 3 that were produced by either parental type (i.e. plants with GHIS of 0 or 6) were considered to be F_1 hybrids. Compared to the number of seeds surveyed, the actual proportion of F_1 hybrids produced within the cohort examined in each population was low (Figure 6.1a & b). Two F_1 seeds were produced within the Darkes Forest population (one from a *B. robur* infructescence, the other from a *B. oblongifolia* infructescence), while the Cataract cohort produced only one F_1 seed from a *B. robur* infructescence. This represents 0.14% of the seeds surveyed in the Darkes Forest population and 0.05% of the seeds surveyed in the Cataract population.

Established F_1 plants within the populations produced seed with a full range of the possible genotypes (Figure 6.2). In both populations the majority of seed produced by F_1 plants had GHIS's of 2, 3 or 4. There were also seed with GHIS of 0 or 6, indicating that, based on the three-locus genotype, apparent "parental" plants can be produced by F_1 hybrids.

The production of apparent "parental" seeds was also evident in F_2 and later generation plants (Figure 6.3a to d). A small proportion of the seeds produced by plants with GHIS of 1 or 2 had a hybrid score of 0. Similarly, plants with GHIS of 4 and 5 produced seeds with a GHIS of 6.

There was significant heterogeneity between observed and expected numbers of each GHIS in both populations (Table 6.9). In both populations there were more seeds with a GHIS of 3 and 5 produced than would be expected from the proportion of mature plants with a GHIS of 3 and 5 in the hybrid zones. It was found that there was a lower proportion of seeds with GHIS of 6 than would be expected from the number of plants with a GHIS of 6 established in the population.

Figure 6.2. The GHISs of the progeny produced by the plants with GHIS of 3.

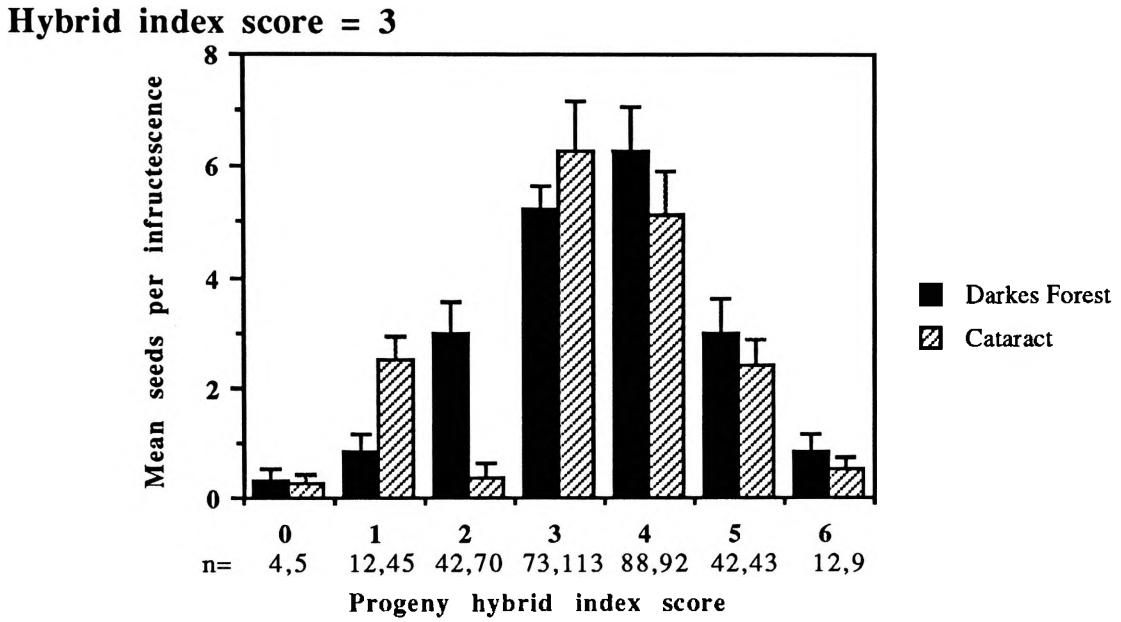


Figure 6.3. The GHISs of the progeny produced by the F_2 and later generation hybrids (or those with GHIS of a. 1, b. 2, c. 4 and d. 5).

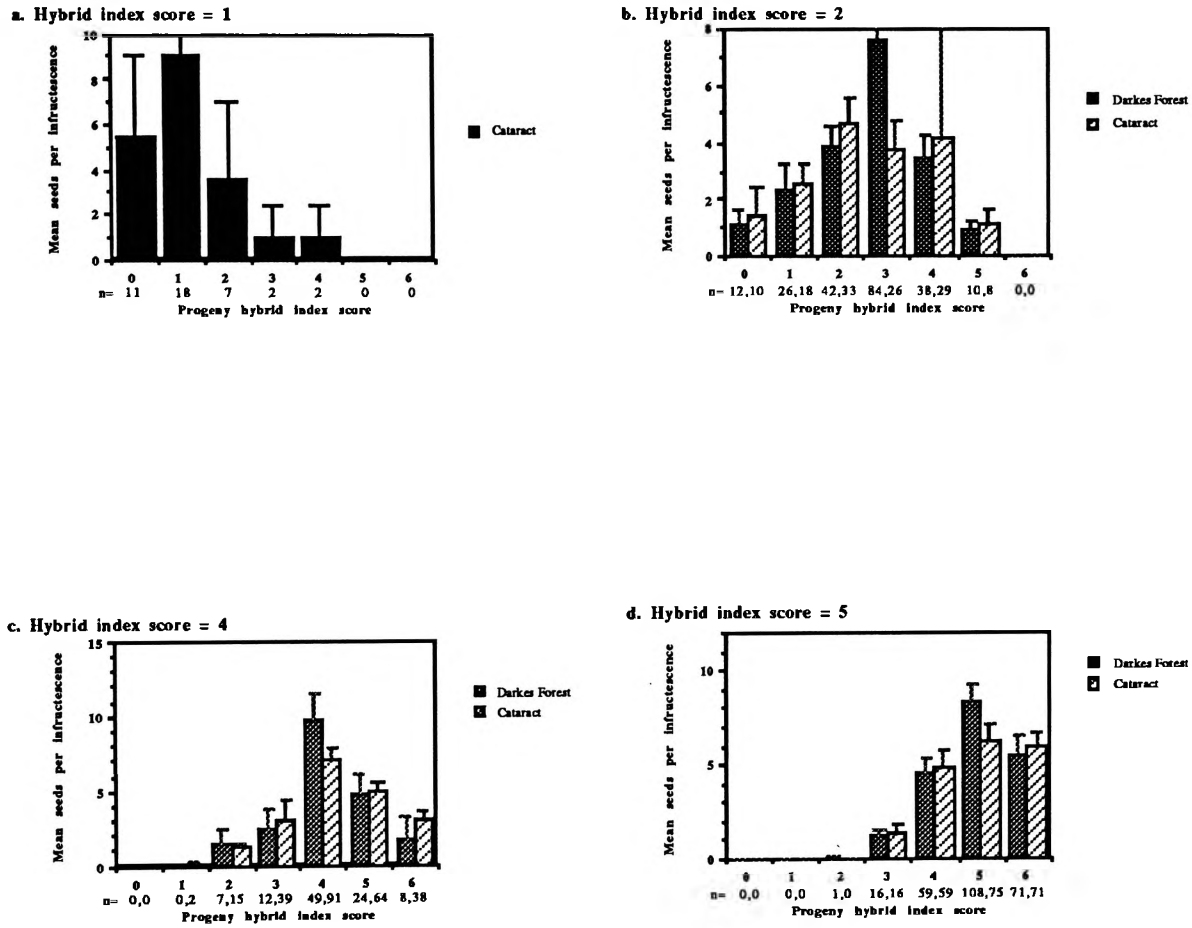


Table 6.9 Chi-square test comparing the observed numbers of seeds with each GHIS and the expected number determined form the proportion of adult plants with each GHIS in a. the Darkes Forest population and b. the Cataract population.

GHIS	Observed	Expectation
a. Darkes Forest		
0	28	27.5
1	1	3.9
2	13	6.2
3	16	11.6
4	7	14.9
5	16	15.1
6	9	12.6
	$\chi^2 = 17.14$	**0.01>p>0.005**
b. Cataract		
0	61	64.3
1	5	6.1
2	7	8.1
3	23	13.1
4	18	18.5
5	18	14.7
6	4	10.7
	$\chi^2 = 13.1$	**0.05>p>0.025**

There was significant heterogeneity between the expected and observed number of seed produced as a result of pollination by intraspecific, interspecific and hybrid pollen for both species in both populations, predicted using flowering synchrony. A more reliable prediction of the number of seeds with GHIS of 3 produced within the population is generated by considering the flowering synchrony of the plants within each hybrid zone, because the production of hybrid seed may vary within and between flowering seasons. Signifcant heterogeneity was detected between the expected and observed number of progeny produced as a result of intraspecific and interspecific crosses and backcrossing. Many more F_1 hybrid seed were expected than observed. More pure *B. robur* seeds (produced from RxR cross), while less pure *B. oblongifolia* (from OxO cross) were produced than was expected (Table 6.10). There was also significant heterogeneity between the observed number of seed with GHIS of 3 and the number predicted, estimated by the combination of flowering synchrony, proportion of types within the pollen pool at these times and the proportion expected with random mating (Table 6.11).

6.3.8. Laboratory germination

The seed bisected to allow electrophoresis and viability assays had a greater than 80% germination success (Figure 6.4). Although seeds with hybrid scores of 3 and 5 were the least viable, viability did not vary significantly among genotypic classes (χ^2=0.347, p=0.999).

6.3.9. Field establishment success

Germination rates in the field were far lower than under laboratory conditions. Of the 900 seeds planted, only 4.2% germinated and grew. Most of these were seed from *B. robur* infructescences. 9.3% of the total number of *B. robur* seed planted had established after 12 months, while only 1.7% of both hybrid and *B. oblongifolia* seed germinated during this time (Figure 6.5). Most of the successful seeds were established within the *B. robur* region of the site (Figure 6.5). Indeed, more *B. oblongifolia* seeds germinated in the *B. robur* region than in the *B. oblongifolia* region.

Table 6.10 Chi-square test comparing the seeds produced as a result of inter-specific, intraspecific and hybrid crosses for each species and the expected number estimated from the proportion of each type of pollen in the pollen pool (estimated using the data in Chapter Four). Tests were carried out for a. Darkes Forest *B. oblongifolia*, b. Darkes Forest *B. robur*, c. Cataract *B. oblongifolia* and d. Cataract *B. robur*.

Cross	Observed	Expected	
a. Darkes Forest *B. oblongifolia*			
O x R	4	42.9	
O x H	132	85.4	
O x O	293	300.7	
		χ^2 = **60.9**	**p<0.001**
b. Darkes Forest *B. robur*			
R x R	421	356.4	
R x H	10	58.9	
R x O	1	16.6	
		χ^2 = **62.1**	**p<0.001**
c. Cataract *B. oblongifolia*			
O x R	2	26.2	
O x H	108	50.5	
O x O	188	221.3	
		χ^2 = **92.8**	**p<0.001**
d. Cataract *B. robur*			
R x R	943	658.0	
R x H	33	257.4	
R x O	1	61.6	
		χ^2 = **378.7**	**p<0.001**

Table 6.11 Chi-square test comparing the number of seeds with GHIS of 3 produced by parental plants (i.e. those with GHISs of 0 and 6) and all other GHISs pooled. The expected proportions were estimated from a combination of the weeks of flowering synchrony, the proportion of each type of pollen in the pollen pool (estimated using the data in Chapter Four), and the proportion of seed with GHIS of 3 expected to be produced under random mating. The maternal GHIS, the GHIS of the progeny and the expected and observed number of seed with GHIS of 3 and the pooled number of seeds with all other GHISs are given for a. Darkes Forest GHISs of 0 and 6, b. Cataract GHISs of 0 and 6.

GHIS of maternal plant	GHIS of progeny	Expected	Observed
a. Darkes Forest			
0	3	79.5	1
	rest	352.5	431
	$\chi^2 = 95.0$	p<0.001	
6	3	28.6	1
	rest	145.4	173
	$\chi^2 = 31.9$	p<0.001	
b. Cataract			
0	3	235.7	1
	rest	741.3	976
	$\chi^2 = 308.0$	p<0.001	
6	3	41.3	0
	rest	35.7	77
	$\chi^2 = 89.1$	p<0.001	

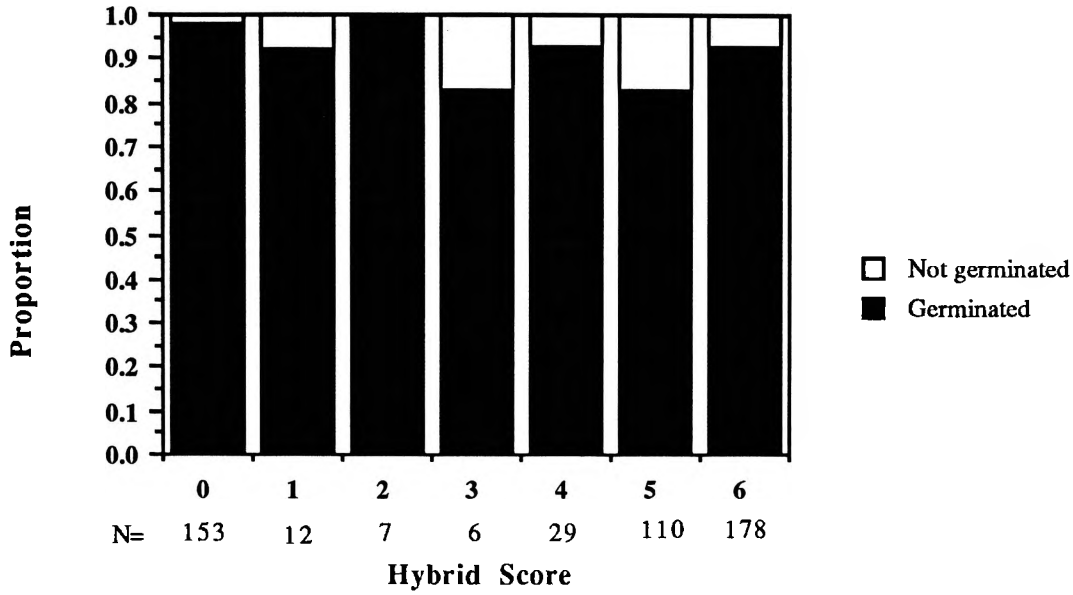


Figure 6.4. Proportion of seeds within each GHIS that successfully germinated

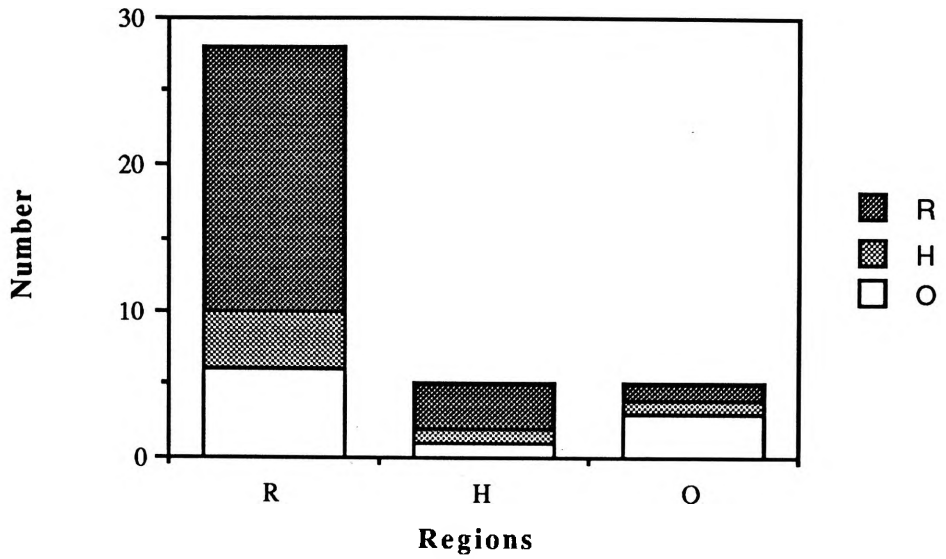


Figure 6.5. The number of seeds germinated under field conditions. The column graph indicates the number of seeds of each type (*B. robur*, hybrid or *B. oblongifolia*) to have successfully germinated and grown into a seedling in each region, designated as R (essentially a pure *B. robur* stand), H (hybrid zone) and O (pure *B. oblongifolia* stand). 100 seeds each of *B. robur*, hybrid and *B. oblongifolia* were planted in each region (a total of 900 seed originally planted in the burnt area).

6.4. Discussion

The results presented in this Chapter indicate that the production of *Banksia robur/B. oblongifolia* hybrids is lower than expected, based on the number adult hybrid plants present in the population. The suggestion of assortative mating, as well as perhaps some selection against hybrids at either the post-zygotic or post-dispersal phase of the life-cycle seem to be contributing factors in the prevention of hybrid formation.

6.4.1. Pollination experiment

The extremely low levels of pollen tube growth and the high levels of fungal infection recorded in the experimental pollination certainly suggested that resultant seed set may be low. The fact that there was no difference in the number of styles colonized by fungus between treatments indicates that fungal growth is not related to treatment, but may be related to environmental conditions. The year in which the experiment was conducted had an unusually high rain-fall (Figure 6.6). The combination of high precipitation and the plastic fly mesh covering the inflorescences to prevent pollinator visitation may have produced high humidity surrounding the inflorescences, conducive to the growth of fungus with the style transmission tissue. Carthew (1993b) reported high fungal growth within styles, and suggested that pollen tube growth was prevented by fungal infection. This excessive fungal growth may partly explain the relatively low seed set of the experimental replicates.

The reasons for the generally low conversion of flower to fruit in *Banksia* are still unknown (Ayre & Whelan 1989). Many of the attempted hand pollination experiments in *Banksia* have also produced very low seed set (Paton & Turner 1978; Carthew 1991; Goldingay *et al.* 1991). However, the low produced in this study were within the range (albeit on the lower end) of natural seed set (reported in Chapter Four, Table 4.6). Indeed, there were a number of instances in the observations of natural seed set, where less than 5% of a particular group of inflorescences formed follicles, while the number of follicles formed by each infructescence was occasionally fewer than twenty.

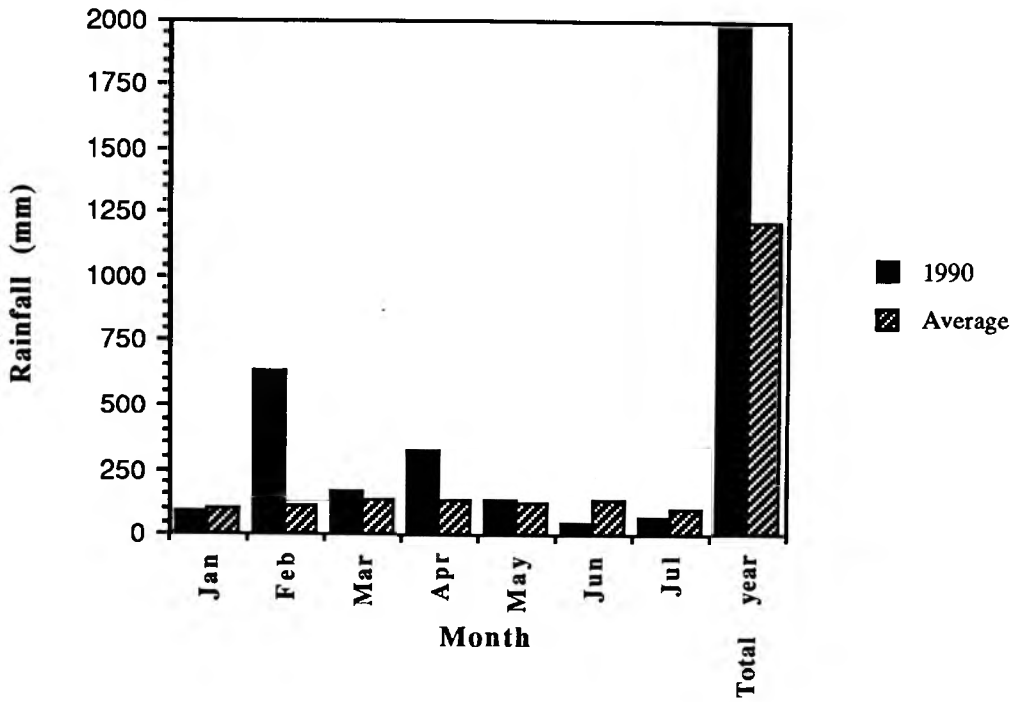


Figure 6.6. A histogram of the total rainfall in each month between January and July 1990 inclusive, and the total rainfall in 1990, with the corresponding overall average of each category. Source: Weather Bureau.

6.4.2. Mating system

The means of the single-locus and multi-locus outcrossing rates for *Banksia oblongifolia* were high compared with estimates obtained for many other plant species (compiled by Schemske & Lande 1985), and comparable to those obtained for other species of *Banksia* (Scott 1980, Carthew *et al.* 1988, Coates & Sokolowski 1992). However, the mean of the single-locus estimates in the Darkes Forest population, and both multi-locus estimates were significantly less than 1, suggesting that a detectable proportion of the progeny were produced by selfing. At face value, the experimental pollination supported the conclusion that *B. oblongifolia* is predominantly outcrossed though with a detectable level of selfing, for although the outcrossing treatments were relatively more successful, seed set was achieved in one selfing treatment in *Banksia oblongifolia.* These results are similar to the level of success of self-pollination treatments in some other studies attempting hand pollination using *Banksia* (Goldingay & Whelan 1990, Carthew 1991, Goldingay *et al.* 1991). However, the results of the experiment do need to be interpreted with extreme caution. The seed set achieved was so low, that it was possible that the results were achieved by chance and not through any maternal choice mechanism.

The flowering phenology survey conducted on *Banksia robur* and *B. oblongifolia* suggest that there are periods within the flowering season where the pollen pool is dominated by the pollen of the other species (Chapter Four). However, as there may be some portion of the progeny of *B. oblongifolia* produced by selfing, self-fertilization may occur more commonly than hybridization. Indeed, the proportion of hybrid progeny produced by these species (discussed below), suggests this to be the case. This is unlike the situation described by Cruzan *et al* (1994) for *Iris*, where hybridization within the genus is thought to occur, because inter-specific pollen is thought to be more successful than self pollen. In *Banksia oblongifolia*, however, caution should be exercised when ascribing to self-fertilization a value of t that is significantly less than one, as detectable outcrossing may be limited by the small amount of allelic variation found in the species.

6.4.3. Assumptions of the mixed mating model

The mixed mating model requires that several assumptions be met: that the alleles must segregate in Mendelian ratios, there must be no association between the genotypes at different loci, and the frequencies of the alleles within the pollen pool are distributed randomly throughout the population of mature plants sampled (Brown *et al.* 1975, Clegg 1980, Hamrick & Schnabel 1985). The results of the hand-pollination experiment and the genotype frequencies of the natural seed set were used to determine if these assumptions were met in this system.

6.4.3.1. Mendelian inheritance

The hand-pollination experiment was used to determine the pattern of inheritance of the alleles in *B. oblongifolia.* Three of the four infructescences produced showed genotype ratios expected under Mendelian inheritance. However, the seed from the infructescence with the largest number of progeny showed significant deviations from Mendelian segregation at all loci. These differences between infructescences may simply have been because the sample sizes were too small. Mulcahy & Kaplan (1979) report that in order to be 90% confident of detecting small deviations from Mendelian inheritance a survey of in the order of 1000 seeds per individual is required. Although the number of seed surveyed in a hand pollination experiment is restricted by the actual number of seed produced, existent deviations may have been masked by the small number of seeds available.

While Mulcahy and Kaplan (1979) argue that deviations from Mendelian ratios are difficult to detect, non-random segregation is not uncommon (e.g. Zamir & Tadmor 1986, Lee & Ellstrand 1987, Pham & Bougerol 1993), and indeed has been found in *Banksia spinulosa* by Carthew (1991). These deviations are thought to be the result of selection at some stage of the plant life-cycle: although zygotic abortion has been suggested to be a cause in some systems (e.g. Pham & Bougerol 1993), late-acting prezygotic selection is thought to control the observed levels of self-fertilization and out-

crossing in *Banksia* (Carthew 1991, Goldingay *et al.* 1991), and these processes are likely to also control the genetic identity of the progeny (Mulcahy & Kaplan 1979).

6.4.3.2. Linkage disequilibrium

Associations between loci may result in non-random inheritance of alleles. Significant linkage disequilibrium between loci is found in predominantly self-fertilizing populations (Brown 1979). Previous studies of *Banksia* have also yielded significant associations between loci in predominantly outcrossing species (Scott 1980, Carthew 1991). The two valid tests of significant deviations from linkage equilibrium in the progeny from pure populations of *B. oblongifolia* may signify linkage of loci, but may also be the result of other processes, such as drift or genetic subdivision of the population (Waller & Knight 1989). If the linkage is real, caution must be exercised in the interpretation of the outcrossing rate, as the significant proportion of selfing detected in *B. oblongifolia* may be due to linkage between loci.

Associations between loci in species within a hybrid zone are expected (Szymura & Barton 1986), due to the continual influx of parental genotypes from "pure" regions. Strong linkage disequilibrium was detected between pairs of loci in adult *B. oblongifolia* plants within the hybrid zone, in Chapter Three, using Hill's estimate of linkage disequilibrium, while the association between loci in the progeny, determined in this Chapter, was not as consistent nor as strong. This change in the degree of association between progeny and adult plants may indicate that selection against hybrid progeny may be occurring at the post-dispersal stage. This is supported by the field planting trials described in this Chapter (see discussion below), and partially supported by the hand-pollination experiment, which suggested that there was no obvious selection at the pre-zygotic stage. However, caution should be used in making a direct comparison between parental plants and their progeny when two different measures of linkage disequilibrium are used.

6.4.3.3. Pollen pool heterogeneity and self-fertilization

Heterogeneity between plants in the pollen type that sires the seed at each locus may indicate selection at the gametic level (Clegg 1980), but could be due simply to spatial heterogeneity within the population, temporal heterogeneity in the pollen pool (Stephenson 1982, Fripp *et al.* 1987, Sampson *et al.* 1990), or a significant but variable level of self-fertilization (Brown *et al.* 1975). The mean fixation index of three of the four stands of *B. oblongifolia* surveyed inferred that there is some selfing occurring in this species, but these values were not comparable to the inbreeding coefficient, indicating that these populations are not in inbreeding equilibrium.

There were fewer instances of significant departures of F from 0 than of t from 1 among the loci within the stands surveyed. Results where t is significantly less than 1, but F is also significantly greater than 0 can arise when there is a component of self fertilization within the progeny array, or there is significant heterogeneity in the pollen pool between plants (Brown *et al.* 1975). Most loci exhibited significant heterogeneity in the pollen pool in both pure and hybrid zone stands of *B. oblongifolia* and the hybrid zone stands of *B. robur*, indicating that, in this system, closely related plants pollinate each other more frequently than would be expected if there was random mating occurring in the population.

Preferential pollination of plants by intraspecific pollen was supported by the significantly larger proportion of seed produced as a result of intraspecific pollination than would have been expected, predicted from the proportion of pollen types within the pollen pool at any one time. The flowering phenology survey conducted in Chapter Four suggested that interspecifc pollination should occur with greater frequency than was detected by the proportion of hybrid progeny in the seed assayed. This result, therefore, supports the idea of assortative mating and/or selection against hybrid progeny.

6.4.4. Hybridization

6.4.4.1. Artificial hybridization

A small resultant seed set from the pollination experiment made it impossible to conclude much about the level of hybridization possible within these species. The failure of any of the interspecific pollinations within the experiment to set seed may have been as a result of insufficient pollen transfer because of pollination technique or interspecific pollen availability. The lack of any difference between the treatments in the amount of pollen tube growth suggests that the interspecific treatment had an equivalent probability of fertilizing the ovule as did the other treatments. Shore & Barrett (1984) demonstrated that there is a threshold number of pollen grains that need to be deposited before the first seed is set, and a second threshold before the full seed set is achieved. This threshold effect was likely to have occurred in this hand-pollination experiment. Secondly, hybrids may be selected against at some prezygotic level, but the few that progress through the selection process, are as fit and vigorous as the parental species. There was no evidence of abnormal growth of pollen tubes within the upper portion of the style (as was evident in Sedgley 1983, Ellis *et al.* 1991, Fuss & Sedgley 1991) and the fact that no significant difference was detected in pollen tubes between species suggests that if selection against the hybrid progeny is occurring, it is at a fairly late prezygotic stage (Seavey & Bawa 1986, Wiens *et al.* 1987). This finding concurs with the conclusions of previous studies on *Banksia* (Lewis & Bell 1981, Carthew 1991, Goldingay *et al.* 1991).

6.4.4.2. Natural hybrid seed set

As many as 75% more hybrids were established within the hybrid zone than was expected from the number of hybrids produced naturally in a cohort. This discrepancy may be explained by one of two hypotheses: (i) the plants with F_1 genotype may be in fact later generation hybrids; or (ii) the hybrid zone and the hybrid are of considerable age, and are the result of hybrid recruitment over several cohorts and/or generations.

Supporting the first hypothesis is the large number of seeds with the genotype typical of F_1 hybrids, present in F_1 or later generation plants. This indicates that once the plant is established, its pedigree is difficult to determine simply from its genotype. In favour of the second hypothesis, the age of individual *Banksia* plants is difficult to determine because of their resprouting capabilities. The plants in this study could potentially be ancient: there has not been a fire in either site for at least ten years, so the youngest plants present in the populations would date from the last fire, while many of the other individuals may be much older. Within the population, therefore, there are potentially many generations coexisting, indicating that the hybrids present in the population may may have been formed in more than one cohort.

The other two sets of predictions also greatly overestimated the number of hybrids produced within the hybrid zones, indicating that mating is not random within the hybrid zones and/or there is some barrier to the production of hybrids at the pre-embryonic stage. Greater precision may be achieved by including all possible factors in the estimation of the predicted number: a superior model would include consideration of proportions expected from random mating, the flowering time, the proportion of genotypes within the pollen pool, the proximity of plants to each other and pollinator behaviour. Description of such a model is the next logical step in the explanation of genotype proportions produced by plants within the hybrid zones.

6.4.5. Natural levels of interspecific pollination

The fertility of organisms of hybrid origin has been historically assumed to be low (Baker 1947). The plants in this study that are of hybrid origin (assessed from the genotype) are capable of both mothering and siring. In Chapter Four, infructescence production by hybrid plants were found to be equivalent to that of the parental species, indicating that the hybrids are capable of being a maternal plant. This Chapter provides evidence that hybrids are equally capable of siring seed. The production of seed with hybrid index scores between that of the parent and 3 (that is, 1 and 2 produced by plants with a 0

score, and 4 and 5 by plants with a hybrid score of 6) indicates that pollen viability of plants from the F_1 and later generations may be high, and these plants are also very capable of siring plants. The proportions of F_1 and particularly the F_2 seeds in the progeny tested and the fertility of the F_2 and later generations indicates that the population is continuing to introgress, which has been thought to be the expected outcome of natural hybridization (Anderson 1949, Grant 1981).

6.4.6. Post-zygotic selection

As was indicated by the comparison of the proportion of GHIS's amongst the progeny to those of the parental plants within the populations, a random sample of seed from each cohort is not necessarily established when the seeds are released. The differences in genotypes of the progeny and the adult plants in the population indicate that there may be some selection acting against *B. robur*/*B. oblongifolia* hybrids. Selection can act at many different stages of the plant's development, including at the post-dispersal and post-germination stages. This is particularly true of these species of *Banksia*, which are predominantly bradysporous. This would mean that once a stand of *Banksia* plants has been burnt, seed is released *en masse*. Despite the possession of a wing, the distance *Banksia* seed is dispersed from the maternal plant is limited to little more than the plant's canopy (Abbott 1985). This means there will be a high degree of competition for space from siblings, and if the species is a resprouter, from the maternal plant as well.

In vitro germination indicated that there was no difference in the germination capacity of the different genotypes. Field conditions certainly reduced the number of seed which successfully establish, but this may have been in response to the hostile post-fire conditions: more *B. oblongifolia* seeds germinated within the wetter soils of the *B. robur* region, than in the drier *B. oblongifolia* region. There were, however, more *B. oblongifolia* seeds successful within the *B. oblongifolia* region than either of the other seed types, while hybrid origin seeds did best within the wetter *B. robur* region.

Low total seed germination and establishment in the burnt site may suggest that the recruitment via seed is indeed low, and that the populations are maintained after fire primarily as a result of resprouting.

6.4.7. Hybrid production and establishment

Drake (1980) summarizes the regulation of successful hybrid production and establishment, to the point where the species and hybrids reach some significant evolutionary outcome (be it reinforcement of reproductive barriers or the formation of a new species). Stress, in the form of selection against hybrid progeny, is particularly apparent at the reproductive phases of this scheme, as is proved in the sterility or lowered reproductive capabilities of the hybrids within the genera of many studies (for a summary of some systems, see Hewitt [1989]). For the production of hybrids and their establishment in the population to occur, these stresses need to be overcome. In these species of *Banksia*, the production of hybrids is certainly occurring, albeit at a slower rate than may have been predicted from the numbers established in the field, and the germination of hybrid and later generation seed does not seem to be impeded. Selection at the establishment phase may, be occurring, but this selection may be weak enough to allow F_1 and later generation hybrids to establish over the long time that is available.

Chapter Seven

General Discussion

The hybrid zone formed by *Banksia robur* and *Banksia oblongifolia* seems to be complex in nature and origin. Conflicting evidence suggests differing selection regimes, acting at different stages of the plant's life-cycle, which may be explained by the interaction of association with the environment and apparent hybrid disadvantage. A summary of the major issues addressed in this thesis, and the evidence supporting these issues, is given in Table 7.1. The numerous possibilities under each issue underlines the complexity of the system. Even amongst fairly limited dispersing plants, the hybrid zone described here is amongst the narrowest described, and certainly amongst dicotyledons. Gene flow within the hybrid zone is limited, while its direction is predominantly asymmetrical. Further, this is one of the first mosaic hybrid zones described in the plant kingdom. This situation provided the opportunity to replicate these observations over two independent boundaries, which indicated in this study that observations in only one site may not be representative of the processes occurring in all populations.

7.1. Hybridization between *Banksia robur* and *Banksia oblongifolia*

Morphological and genetic evidence both indicate that hybridization is occurring between *Banksia robur* and *Banksia oblongifolia*, in both of the two sites studied in detail. Furthermore, these species seem to hybridize readily whenever they come into contact, because zones containing plants of intermediate morphology have been observed in other populations near Wollongong, north of Sydney, and surrounding Brisbane.

Reproductive isolation is traditionally the central tenet of species definition (Mayr 1963, Dobzhansky 1970). More recent species concepts, however, define organisms more adequately using ecological and developmental characters, as well as reproductive

Table 7.1. Evidence within the *Banksia robur/Banksia oblongifolia* gathered in this study supporting the issues listed in Table 1.1.

Issue	Hypothesis	Chapters in which addressed	Support for hypotheses in this *Banksia* study
1. Hybridization?	*Phenotypic plasticity?*	Two	NO, hybrids produced by these species are genetic hybrids, and the morphological differences are associated closely to the genotypes, although some characters measured display some signs of plasticity
2. Role of hybridization in speciation	*Reinforcement*	Four & Five	No evidence of reinforcement in flowering time or attraction of specific pollinators. Small shift in the flowering time of *B. oblongifolia* between plants within the hybrid zone and pure stands, which may be environmentally based. There is no evidence disputing speciation by reinforcement in this system. A longer term study is required.
	Character displacement	Four & Five	Flowering time is the only well developed specific mate recognition character. This character separates the species well - there is limited opportunity for inter-specific gene exchange.
3. Cline formation	*Model 1*	Two, Three & Six	Occurrence of backcrossing and introgression confirmed. Hybrids are capable of producing and siring seed.
	Model 3	Three & Six	Some evidence of selection against hybrids from selection coefficients calculated using cline shape parameters. Production of hybrid seeds are low compared to several sets of expectations derived from existent plants, flowering synchrony and proportions expected under random mating.

Table 7.1.continued

	Model 4	Three
	Model 5	Three
	Model 6	Three
4. Origin of hybrid	*Parapatric speciation: Endler (1977)*	Two & Three
	Allopatric speciation: Barton & Hewitt (1981, 1985)	Three & Six

NO. Selection against hybrids does not seem to occur after establishment. Once hybrid plants are established, they seem to compete as well as the parental plants within the hybrid zone.

Parental types are found within specific regions - there is a different set of alleles found in different positions along the cline.

Not quite. Evidence suggests that hybrids are as fit as the parental plants within the hybrid zone.

Zone is probably environmentally dictated. Lack of genetic variation within and between species may suggest that they were recently diverged. The environmental restriction of *B. robur*, and its lack of genetic variation, suggests that it is more specialized and perhaps more recently derived? Very speculative - not enough evidence.

Likely to be tension zone, or at least environmental cline maintained by gene flow and selection against hybrids. More likely to explain disjunct distribution of species.

isolation (Endler 1989, Templeton 1989). Because the definition of a species takes into account, but is not reliant on, reproductive isolation being in place, an essential place to begin the study of the processes leading to speciation is through the determination of the degree of reproductive isolation (both pre- and post-mating) in closely related species (Littlejohn *et al.* 1971, McDonnell *et al.* 1978, Shaw *et al.* 1986, Baker & Baker 1990). For hybridization to take place, there must be at least some synchrony in reproductive phases of the two species, the transfer of male gametes, and successful fertilization. It is expected that the opportunity for gene exchange between the incipient species will be limited and eventually stopped after some time, as synchrony in reproductive characters decreases through reinforcement, character displacement or mate recognition divergence (Butlin 1989).

The field observations of *Banksia robur* and *Banksia oblongifolia* suggest that there is currently adequate opportunity for hybridization. Partial synchrony of flowering times and the indiscriminate foraging behaviour of the likely pollinators makes pollen transfer between the species possible. However, the level of successful hybridization could not be tested, because attempts to produce hybrid seeds by hand-pollination failed. This failure may have been due to general difficulties involved in hand-pollination of *Banksia*, which has been reported by a number of workers (Carthew 1991, Goldingay *et al.* 1991). The small proportion of F_1 hybrids produced naturally in the most recent cohort, however, suggests that hybrid production may be fairly limited under natural conditions anyway.

7.2. Introgression

In the *Banksia robur/B. oblongifolia* system, interspecific gene flow may be primarily through backcrossing. This was suggested by the observations of flowering times, in which there was a greater synchrony in the flowering time between each parental species and the hybrids, than between the two species. Substantial levels of backcrossing were also evident in the genotypes of the progeny from the most recent cohort.

Allopatric populations of the two species were found to be virtually fixed for different alleles, although individuals from allopatric populations of *Banksia oblongifolia* exhibited low frequencies of the "*B. robur*" alleles. Alleles characteristic of one species in the genome of an isolated population of another species, with which it is known to hybridize, has been documented in a number of studies on floral hybridization (e.g. Drake 1980, Arnold *et al.* 1990). The presence of these alleles have been proposed to be due to relict ancestral variation, or past introgression (Sage & Selander 1979, Harrison 1986, Arnold *et al.*1990), and there need not be any morphological evidence of the genetic contribution of the other species (dePamphilis & Wyatt 1990). This was certainly evident in the case of pure populations of *B. oblongifolia*, which were morphologically distinct from the isolated stands of *B. robur*. Which hypothesis best explains the origin of the uncharacteristic alleles in a particular situation can only be tested using independent genetic surveys (e.g. determining both allozyme and DNA variation within the system [Marchant *et al.* 1988]).

Introgressive hybridization may affect a population in one of several ways. Gradual merging of the two gene pools is possible, giving rise to recombinants adapted to a wider variety of niches (Anderson 1953). However, even the production of fertile hybrids will not necessarily make the parental gene pools more similar (Bigelow 1965), because hybridization can eventually reinforce the separation of two species by hybrid disadvantage (Szymura and Barton 1986). In the *Banksia* hybrid zone, introgressed recombinants may have some adaptive advantage in the ecotonal environment where the mixed species stand and the hybrids are found.

7.2.1. Asymmetrical introgression

The results of several aspects of this study suggest that the direction of gene flow between species is asymmetrical. Genotypes of the pure stand populations, the differences in the length and slope of the tails of the clines and the timing of flowering of the two species all suggest that gene flow is primarily in one direction: from *B. robur* to *B. oblongifolia*.

Therefore, F_1 hybrids produced are more than likely to have a *B. oblongifolia* mother than would be expected from random pollination of flowers. This suggests that essentially the zone is a mobile "tension" hybrid zone, as defined by Hewitt (1988). Zones formed in this way will tend to come to rest in regions such as environmental ecotones, an explanation that fits well the region where the *B. robur/B. oblongifolia* hybrid zone described is found. Although this idea is speculative, the estimated direction of gene flow could be inferred from assessment of variation in mitochondrial DNA (or perhaps more appropriately in this case, the chloroplastal DNA) by RFLP or sequencing studies, because the genome of these organelles are normally transmitted solely along the maternal lineage (Avise 1986).

7.3. The *Banksia robur / Banksia oblongifolia* hybrid zone

Most theoretical models designed to examine hybrid zones represent the transition from one species to the other in a simple, monotonic cline, and in most cases this treatment is satisfactory to summarize the change in the trait across the zone. A simple cline, however, could not be fitted to the *B. robur/ B. oblongifolia* hybrid zone. There were many areas of contact, where the species meet within the species' range along the east coast of Australia and, further, there was spatial heterogeneity within the hybrid zone regions in both populations observed, prompting the description of the hybrid zone formed by *B. robur* and *B. oblongifolia* as a "mosaic" hybrid zone (Harrison 1986). There may potentially be many unique consequences of the formation of a mosaic hybrid zone, as opposed to a simple cline, on the dynamics (stability and characteristics) of the zone. For example, an increase in the frequency of contact between the parental species can result in a broader zone of contact, increasing the probability of reproductive isolation through reinforcement (Butlin 1989). There is no evidence within the *B. robur/B. oblongifolia* hybrid zone, however, of reinforcement of reproductive isolation, although, neither is there evidence against it.

The outcome of evolutionary forces within different patches, even between patches of the one species or species complex, may vary (Harrison 1986). This calls for the consideration of each site separately in both the allelic variation and phenotypic characteristics. This study has confirmed that this consideration is essential, as there were differences between the Cataract and Darkes Forest populations in the proportions of genotypes, selection regimes calculated using cline shapes and genetic neighbourhood sizes.

7.4. Selection

A surprising result of this study is the small number of seeds that are of F_1 origin within the natural cohort examined compared to the proportion expected, and the lack of any produced in the hand-pollination experiment. This is surprising because of the high proportion of apparent F1 hybrids established to reproductive age within the natural population. In comparison to the number of adult hybrids apparent in the hybrid zone populations, the proportion of hybrids seed produced naturally was significantly lower than expected. Even the limited flowering synchrony between *B. robur* and *B. oblongifolia* predicted there would be a higher proportion produced. Some of the hypotheses explaining this have been discussed previously (Chapter Six), but one of the most likely scenarios is the combination of selection against the hybrids and the longevity of the plants involved. The extremely high selection coefficients calculated for the centre of the cline, coupled with the low estimate on the alleles outside the zone, suggests that the selection of hybrids formed by the union of *B. robur* and *B. oblongifolia* is indeed high.

As the level of seed set obtained in the pollination experiment was similar to that expected obtained in surveys of natural seed set, it may be suggested that the level of natural hybridization involving *Banksia robur* and *B. oblongifolia* may be fairly low, agreeing with some other attempts at interspecific hand pollination (Borgen 1976, Bernhardt & Calder 1981). Some other published artificial interspecific crosses contradict this, finding

that hybrid production at at least the embryonic and later stages is high (e.g. Littlejohn *et al.* 1971, Howard 1986, Levin & Bulinska-Radomska 1988), although these hybrid progeny may be selected against at a later stage of development.

7.5. Gene flow and speciation

Just as the extent of gene flow within a population of plants of the one species can potentially effect the genetic substructure of the population (Loveless & Hamrick 1984), so the pattern of gene exchange between hybridizing species can dictate the homogeneity of the species' genome (Arnold *et al.* 1992). Gene flow between closely positioned plants can result in groups of related individuals of the one species, particularly if gene flow via seed dispersal is limited. Even if hybridization readily occurs, limited gene flow will result in limited opportunity for gene exchange between species and a very slow progression of alleles across the zone of hybridization. Limited dispersal of genes within the *B. robur*/*B. oblongifolia* hybrid zones would result in fairly short gene dispersal distances and the predominance of intraspecific mating, despite the apparent opportunity for hybridization.

Estimates of dispersal distance from different methods used in this thesis are conflicting, but are at most about 500 m, and may be as little as 10 m. Restricted movement of pollen is also suggested by the small neighbourhood size and the heterogeneity in the pollen pool frequency of the progeny, which indicate that pollination is potentially within closely situated, closely related groups of plants. Pollination amongst closely related plants potentially provides the opportunity for spatial variation in the genetic structure of the system, and, therefore, the maintenance of differentiation between *B. robur* and *B. oblongifolia*. This situation is confirmed by the genotypes of progeny resulting from natural seed set. Most of the seeds produced by *B. robur* inflorescences are *B. robur* seed, while there are mainly *B. oblongifolia* progeny produced by *B. oblongifolia*, suggesting that despite the lack of pre-zygotic reproductive barriers, the two species are largely remaining genetically intact.

If, on the other hand, the estimates of gene dispersal calculated from the cline shape parameters are correct, the hybrid zones are subject to relatively large distance gene flow. Nonetheless, in spite of what appears to be extensive and random gene flow, it is still possible for the genetic differentiation apparent in *B. robur* and *B. oblongifolia* to occur within a continuous population (Endler 1973, 1977). Early studies by Thoday & Gibson (1962) on *Drosophila* have suggested that differentiation of a character can occur within the one population after several generations of selection, although this genetic isolation disruptive selection has been impossible to repeat (de Meeûs *et al.* 1993). However, Endler (1977) modelled the formation of a cline within a continuous population after a number of populations through drift. These clines proved unstable, unless there was some selection for these genotypes, as a result of, for example, adaptation to certain different environments. A more recent model (de Meeûs *et al.* 1993) indicated that sympatric speciation is possible as the result of colonization of a particular habitat by one genotype, under selection that is both frequency and density dependent, allowing the regulation of the population within the habitat. Dubbed "soft selection" (Levene 1953), this model is capable of explaining the *B. robur*/*B. oblongifolia* complex, as the selection will automatically improve the habitat specialization, or the isolation, of the types, and result in deficiencies of the heterozygotes, which seems to be in progress, as evidenced by the selection against hybrids within the cline.

While most hybrid zones are thought to have arisen as a result of secondary contact, it is difficult to tell the difference between instances of secondary and primary intergradation, unless observation of the zone is within a few hundred generations of contact (Endler 1977). However, it is difficult to determine the age of the contact zone between *Banksia robur* and *B. oblongifolia*, because of their life-history. The recruitment strategy typical of *Banksia*, and characteristic of *Banksia robur* and *B. oblongifolia*, is that individual plants will resprout and release their seed after fire (George 1981, 1987), thereby creating a population of plants of many different age (or generation) classes. This means that any

hypotheses posed as to the origin of the complex need to be made in the context of the overlapping generations exhibited by the species.

Many tension zones (which is a partial explanatory model for the *B. robur/B. oblongifolia* hybrid zone) described in the literature are thought to have arisen as a result of secondary contact (Hewitt 1989). The presence of the *B. robur* allele in the pure stand *B. oblongifolia* genome could be explained as a result of previous contact, which had subsequently broken. The small proportion of these alleles in the *B. oblongifolia* genome suggests that the supply of *B. robur* alleles were not constant. The disjunct distribution of *B. robur* along the east coast of Australia may support this hypothesis, in that it is highly unlikely for the species to evolve more than once along more 1000 kilometres of coast line.

Alternatively, there are several aspects of the *Banksia robur/B. oblongifolia* system suggest that *B. robur* is recently derived from *B. oblongifolia.* At the three loci observed to be polymorphic for *B. oblongifolia, B. robur* are fixed for the least common allele at each locus. This may suggest that the *B. robur* alleles are more suited to the waterlogged soils or along water courses, where *B. robur* is primarily found (these habitats are described by Keith & Myerscough [1993]). It can be imagined that certain alleles may be advantageous in situations of chronic water-logging, and the fact that *B. oblongifolia* is tolerant of waterlogged soil to some extent suggests that tolerance is inherent in its genome. For example, flooding and the subsequent drop in oxygen available to the root system, has been known to result in variation in the activity of ADH, because of its central role in the fermentation process (Chan & Burton 1992). Variants on the *B. oblongifolia* genome that are advantageous to water-logging may be more successful and proliferate in chronically water-logged soils. It is expected that a population of the recently formed variants have reduced genetic diversity, as they are often formed as a result of founder effects and genetic drift (Abbott 1992). This reduced diversity is characteristic of *B. robur.*

7.6. Conclusion

While this study provides evidence that both environmental association and hybrid disadvantage may contribute to the maintenance of the *Banksia robur/B. oblongifolia* hybrid zone, improved results in many aspects of this study may provide the impetus for preference of one model over the other. Improved results in the experimental pollination may not only provide an insight into the potential levels of hybridization in this complex, but detailed analysis of pollen tube growth may also uncover selection at a pre-zygotic stage, as yet undiscovered. Further investigation of environmental heterogeneity may also indicate the nature of the correlation between water-logging and genotype. Transplant experiments should account for the environmental heterogeneity in greater detail and conducted over greater areas and a longer time span. The little genetic variation among and between the species made genotypic analysis difficult and less stringent. Since allozyme electrophoresis could not provide sufficient variation, more advanced DNA techniques may be more useful in genotyping the plants in this system. This would also enable genetic information to be obtained from plants where limited material is available (through PCR techniques). These types of questions would be the logical next step in the further investigation of this system.

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Appendices

Appendix 2.1

Summary of the electrophoresis systems tested using the *B. robur*/*B. oblongifolia* complex. The table lists the total number of enzymes tested, the associated Enzyme Commission reference numbers, buffer system used to screen enzymes, the number of loci detected and the resolvable variation useful in separating the two species. The numbers used under the "Buffer Used" column refer to the buffer numbers used in Selander *et al.* (1971). The others not used in Selander *et al.* (1971) are: Hist.: a Histidine buffer; Pou.: Poulik buffer; TC6: Tris citrate buffer pH=6. Under the "Loci" column, the number of resolvable loci using seed and seedling assays are given for each enzyme. The loci numbers preceded by "P=" indicates the number of loci resolved using pollen. The polymorphic loci are indicated in the "Variation" column: the loci that were polymorphic are numbered according to relative mobilities, - indicates that no loci were polymorphic, "Unres." indicates that polymorphisms were apparent, but not consistently resolvable.

Enzyme	E.C. Number	Buffer Used	Loci	Variation
Aconitase	4.2.1.3	2,5,6,9	1	-
Acid Phosphotase	3.1.3.2	6	1	-
Alcohol Dehydrogenase	1.1.1.1	5	2 P=4	Adh_1 & Adh_2 Unres.
Aldehyde Oxidase	1.2.3.1	5	1	-
Aldolase	4.1.2.13	6,9, Hist.	1	-
Alkaline Phosphatase.	3.1.3.1	6	-	-
Arginine Phosphokinase	2.7.3.3	9	-	-
Catalase	1.11.1.6	6, Pou.	1	-
Creatine Kinase	2.7.3.2	9	-	-
Diaphorase	1.6.4.3	9	-	-
Esterase	3.2.1.2	6, Pou.	-	-
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	5,6,9,TC6	3 P=2	- -

Glutamate Dehydrogenase	1.4.1.2	5, TC6,9	2 P=2	*Gdh*1 & *Gdh*2 -
Glutamate oxaloacetate transaminase	2.6.1.1	5	-	-
Hexokinase	2.7.1.1	5	1	-
Isocitrate Dehydrogenase	1.1.1.42	2,5,6,Hist.	2	-
Lactate Dehydrogenase	1.1.1.27	9	1	-
Leucine Aminopeptidase	3.4.11.1	2, 5, 6, Pou,Hist	1	-
L-leucyl-L-glycylglycine peptidase	3.4.13	6, Pou	3	Unres.
L-leucyl-L-tyrosine petidase	3.4.13	6, Pou.	2	Unres.
L-leucyl-proline peptidase	3.4.13	6, Pou.	1	-
Malate Dehydrogenase	1.1.1.37	2,5,6,9, Hist, Pou.	3 P=3	- -
Malic Enzyme	1.1.1.40	9 5, TC6	1 -	- -
Mannose phosphate isomerase	5.3.1.8	2,5,9, TC6	1	-
Octopine Dehydrogenase	1.5.1.11	5,6,9,TC6	1	-
Phosphoglucose isomerase	5.3.1.9	2,5,9, TC6,Pou,Hist.	2 P=2	Unres. Unres.
Phosphoglucomutase	2.7.5.1	9, Pou., Hist.	1 P=1	Unres. Unres.
Shikimate Dehydrogenase	1.1.1.25	5,6,9,Pou.	-	-
6-Phospho-glucose Dehydrogenase	1.1.1.44	5,9, TC6	3	-
Sorbitol Dehydrogenase	1.1.1.14	5,6	1	-
Superoxide Dismutase	1.15.1.1	2,5,6,9, TC6, Pou.	2	*Sod*1

Appendix 2.2

One-factor ANOVA for each morphological character, measured from plants within the *B. robur* and *B. oblongifolia* pure stands. The levels within the factor are the three hybrid indices found in the pure stands, 0, 5 and 6. DF - degrees of freedom, F - F statistic and P - probability. NS indicates that no significant difference was detected (P>0.05); * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001.

Character	Source of Variation	DF	Mean Square	F	P
a.Leaf length	Index	2	359.63	233.63	****
	Within Index	43	1.54		
b.Leaf width	Index	2	104.35	95.40	****
	Within Index	43	1.09		
c.Leaf to widest point	Index	2	179.51	23.70	****
	Within Index	43	7.57		
d.Petiole length	Index	2	5.64	105.25	****
	Within Index	43	.05		
e.Petiole width	Index	2	0.14	184.55	****
	Within Index	43	.001		
f.Serrations	Index	2	0.36	1.62	NS
	Within Index	43	0.23		
g.Recurved veins	Index	2	1104.74	45.55	****
	Within Index	43	24.25		
h.Marginal veins	Index	2	2549.68	48.81	****
	Within Index	43	52.23		
i.Leaf area	Index	2	22860.94	141.95	****
	Within Index	43	161.06		
j.Inflorescence length	Index	2	2.00	0.97	NS
	Within Index	43	2.05		
k.Inflorescence width	Index	2	1.34	26.20	****
	Within Index	43	0.05		

l. Rows of flower pairs	**Index**	2	13.19	1.02	NS
	Within Index	43	12.98		
m.Flower pairs per whorl	**Index**	2	33.97	6.31	*
	Within Index	43	5.39		
n.Style length	**Index**	2	1.24	18.226	****
	Within Index	43	.07		

Appendix 2.3

One-factor ANOVA for each morphological character, measured from plants within the two hybrid zones surveyed. The single factor used was the hybrid indices of the plants measured. For characters a-j, all hybrid indices are included, while for characters i-n, hybrid index of 1 is excluded as no inflorescences were available from these plants. DF - degrees of freedom, F - F statistic and P - probability. NS indicates that no significant difference was detected (P>0.05); * - P≤0.05; ** - P≤0.01; *** - P≤0.001; **** - P≤0.0001.

Character	Source of Variation	DF	Mean Square	F	P
a. Leaf length	Index	6	358.71	76.71	****
	Error	132	4.67		
b. Leaf width	Index	6	96.26	112.34	****
	Error	132	0.857		
c. Length to widest point	Index	6	122.69	59.72	****
	Error	132	2.05		
d. Petiole length	Index	6	5.89	136.05	****
	Error	132	0.04		
e. Petiole width	Index	6	0.133	77.72	****
	Error	132	0.002		
f. Serrations	Index	6	0.395	2.86	*
	Error	132	0.138		
g. Recurved veins	Index	6	792.296	24.51	****
	Error	124	32.326		
h. Marginal veins	Index	6	2099.3440	18.82	****
	Error	132	111.556		
i. Leaf area	Index	6	24677.837	99.94	****
	Error	138	246.923		
j. Rachis length	Index	5	29.218	5.795	****
	Error	75	5.042		

k. Rachis width	**Index**	5	2.302	14.809	****
	Error	75	0.155		
l. Rows of flower pairs	**Index**	5	63.445	2.998	*
	Error	75	21.162		
m. Flower pairs per whorl	**Index**	5	116.022	14.033	****
	Error	75	8.268		
n. Style length	**Index**	5	0.373	3.426	**
	Error	75	0.109